Expression Profiling in Pakistani Cotton *Bt* Genotypes

By

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Cott-Weed control
Genotypes
Bacillus thuringiensis

Expression Profiling in Pakistani Cotton *Bt* Genotypes

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"In the name of ALLAH The Most Gracious and The Most Beneficial"

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Dated: _____

FINAL APPROVAL

It is certified that we have read the final thesis entitled **"Expression Profiling in Pakistani Cotton *Bt* Genotypes"** submitted by **Mr. Zulqarnain Manzoor** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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A thesis submitted to Department of Bioinformatics and Biotechnology,
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This humble effort is

Dedicated

To

My beloved respected

Parents, Teachers, Brothers, Sisters and Friends

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Expression Profiling in Pakistani Cotton *Bt* Genotypes"** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

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ABBREVIATIONS

Abbreviations	Definitions
≈	Almost equal to
δ	Delta
-ve	Negative
+ve	Positive
A	Alpha
B	Beta
$(NH_4)_2 SO_4$	Ammonium sulfate
°C	Degree centigrade
µg	Microgram
µg/g	Microgram per gram
µl	Microliter
Bp	Base pair
Bt	*Bacillus thuringiensis*
CaMV35s	*Cauliflower Mosaic Virus* 35S
CFUs	colony farming units
cm	Centimeter
CO_2	Carbon dioxide
Cry	Crystal
CTAB	Cetyl trimethyl ammonium bromide
DAE	DNA After Enrichment
DAS	Days after sowing
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene Diamine Triacetic Acid
EF-1-alpha	Elongation factor-1 alpha
ELISA	Enzyme linked immune sorbent

	assay
F	Forward
GDP	Gross domestic product
GM	Genetically modified
GMOs	Genetically modified organisms
GUS	β-glucuronidase
H	Hour
ICPs	Insecticidal crystal proteins
IPM	Integrated Pest Management
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LPS	Lipopolysaccharides
mg	Milligram
$MgCl_2$	Magnesium chloride
Min	Minute
ml	Milliliter
mM	Millimolar
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
Npt-II	Neomycin phosphotransferase II
PCR	Polymerase Chain Reaction
PHYB	phytochrome B
ppb	Part per billon
R	Reverse
rpm	rotation per minute
Sad1	Stearoyl-ACP desaturase
SWR	Standing-wave ratio
TBE	Tris/Borate/EDTA

T.SOD	Trypsin superoxide dismutase

ABSTRACT

Using soil bacterium *Bacillus thuringiensis* (gram positive) as a source for insecticidal gene (*Bacillus thuringiensis)* to engineer/modify cotton. *Cry* (Crystal) genes such as *Cry1Ac, Cry1F* and *Cry2Ab* are found in *Bt* cotton that are highly effective against several cotton insects like *Lepidoptera* and acts feasibly for both, human and environment. *Bt* insecticidal factors does not act as continues, so some significant evidence recommends that highly measured *Bt* expression is needed for the efficiency of *Bt* cotton. To fulfill the resistance requirements in targeted pest complex, functionalization of *Bt* genes plays a vital role and needs better understandings to know about the mechanism and proficiency of *Bt* factors. The current study was planned to assess gene expression profiling in Pakistani cotton *Bt* genotypes under glasshouse conditions which includes detection of the types of *Bt* proteins expressing in each plant genotype, confirmation of *Bt* genes in plant genotypes at molecular level and quantification of *Bt* (*Cry1Ac*) gene expression in transgenic *Bt* cotton. Immunostrip assay and PCR analysis were performed for the detection of *Bt* genes, a total of 72 genotypes (NCVT-1601 to NCVT-1672) were evaluated that exposed the occurrence of only *Cry1Ac* factor, except for four genotypes, among all genotypes. Temperature 25 to 30°C was maintained under glasshouse conditions for cultivation of all the genotypes. On 80 days after sowing (DAS) fresh leaf tissues collection was carried out and ELISA was performed for the quantification of *Cry1Ac* gene expression (*Bt* toxin). Among the selected genotypes, eighteen genotypes (NCVT-1601, NCVT-1603, NCVT-1604, NCVT-1610, NCVT-1618, NCVT-1633, NCVT-1645, NCVT-1647, NCVT-1648, NCVT-1650, NCVT-1653, NCVT-1655, NCVT-1656, NCVT-1659, NCVT-1661, NCVT-1662, NCVT-1664 and NCVT-1672) expressed the maximum level of *Bt* toxin (2.6689, 2.2099, 2.6903, 2.4456, 2.3757, 2.6894, 3.3999, 3.5539, 2.7689, 4.0460, 2.9871, 3.7258, 2.9 862, 5.6074, 5.3202, 4.6184, 3.9601 and 4.1008µg/g, respectively) which ranged from 2.2099 – 5.6074µg/g on 80 *DAS. Twenty-one genotypes expressed the medium level of *Bt* toxin ranged from 1.0410– 1.9826µg/g, seventeen genotypes expressed low level of *Bt* toxin ranged from 0.1605– 0.9765µg/g while twelve genotypes expressed very low level of *Bt* toxin ranged from 0.0145– 0.0895µg/g. These findings

will help in selecting the cotton genotypes with high level of expression for pursuing for varietal development. Since low level expressing genotypes have started developing resistance in insects. This study will also help setting a basic criteria of expression level of *Bt* toxin for cotton crop.

INTRODUCTION

1. INTRODUCTION

Pakistan is the third largest exporter of raw cotton, that is the backbone of textile industry of the world, the fourth largest consumer of this cash crop. Pakistan has the title of fifth largest producer of cotton in the world because 1.3 million farmers (out of total of 5 million) are cultivating cotton over 3 million hectares occupying 15 percent of the cultivable area of the country. Also Pakistan is consuming 30 and 40 percent of final products of cotton. 55 per cent to the foreign exchange earnings and about 10 per cent to GDP of the country subsidized by cotton and its products, that's why Pakistan is known as the largest exporter of the cotton (Economic Survey of Pakistan 2008-09)

This white gold (cotton crop) has a prominent position in Pakistan's local textile and edible oil industry, so serving as a backbone of Pakistan (Khan *et al.*, 2009). Although cotton has very impressive and imperious production but due to expense of other crops it is very difficult to afford more land to produce cotton crop. So efforts should make more to increase the production of this cash crop. Pakistan is growing cotton on the area of 15 percent of arable land, by doing so it subsidizes around 8 and 54 percent of total GDP and export wages of Pakistan respectively (Rehman, 2014).

According to another study Pakistan is keeping 4th and 3rd position respectively in production and consumption of cotton. Pakistan has commercialized only the genetically modified *Bt* cotton crop and from which 16 of its different varieties are covering 2.8 million hectors of land and that are fostered on approximately 85 percent the area of implanted land (Rehman, 2013). It is the most value able and coins crop that fulfills the better wealth for the country in the form of foreign exchange. After the independence of Pakistan, cotton is playing an aggressive role in agriculture, industries, employments and financial solidity and also to minimize the economic problems of the country (Ahmed *et al.*, 2009).

According to a survey report more than 1326 species of insects attacking on cotton crop in the world while it is susceptible to attack more than 15 violent insects like *Lepidoptera* insects like American boll worm (*Heliothis_armigera*), pink boll worm (*Pectinophora_gossypiella*), army bollworm (*Spodoptera_lithura*) and spotted boll worm (*Earius_insulana/vitella*) (Atwal, 2002). Even in subcontinents, it is infected by 162 violent species of pathogens and bug worms (Manjunath, 2004).

As the effect of harmful insect pest is very complex also requires enough knowledge of using the *Bt* gene because the *Bt* gene has very effective role in the growth and protection of the plant according to the variations in time, environment and the age of the plant because of the complexity in *Bt* protein mechanism. The present study was designed to evaluate the expression profiling of *Cry1Ac* gene in 72 *Bt* cotton genotypes (NCVT-1 to NCVT-72) grown under glass house conditions. In some studies, agricultural system has used of comprehensive varieties of highly toxic agrochemicals. Because of some serious environmental and human health problems there is a need to look forward for the biological control trials. Pesticides can solely be used to mimic the crop losses but they also serve to create environmental and human health issues. Cotton crop is also badly infected on different developmental and growth stages by various kinds of worms like, (white fly, aphids, trips etc.) and sucking insect (Boll worm species) that results in the reduction of yield on large scale (Bakhsh *et al.*, 2009).

By introducing new traits to make genetically engineered plants cotton has got an interest in the field. It was suggested that with the introduction of new genes into plants, can make them more active, play an important role in stability of resistance and also make them protective efficient (Chen *et al.*, 2003). The insecticidal genes (*Bt* gene) obtained from gram positive, soil bacterium *Bacillus thuringiensis* were transformed into the genetically transformed cotton (*Bt* cotton) in purpose of achieving the goal. The Lepidopteron pests are well controlled by *Bt* cotton having *Cry* gene(s) like *Cry1Ac, Cry1Ac/Cry2Ab* or *Cry1Ac/Cry1F*, are highly effective in reducing chemical insecticide sprays facilitating the grower as well as environment and also helpful to preserve the population of essential arthropods (Gianessi and Carpenter, 1999; Tabashnik *et al.*, 2002).

The δ-endotoxin (*Cry* Toxin) proteins are reportedly appeared in every part of the plant when genetically changed *Bt* cotton is applied. δ-endotoxins comes under the class of insecticidal crystal proteins (ICPs) formed by *Bt* (Kumar *et al.*, 1996). A large number of insect pest complex and large variety of invertebrates are controlled by these proteins. The process of the solubilization of crystal in the midgut of insect, the process of proteolytic of the protoxin on the midgut proteases, the connecting of *Cry* toxin with the midgut receptors, creating ion channels or pores by the insertion of the toxin into the

apical membrane (Crickmore *et al.*, 1998). The toxin proteins are connected to specific receptors in region of midgut when treated by larvae and this also disturbs midgut epithelium, and finally the larvae's death by the complete toxic effect are included in the mechanism of action of the *Bt* (*Cry*) proteins (Kranthi *et al.*, 2005).

The results of genetically modified cotton crop *Bt* toxins varies and show different statistical numbers when experiments performed in the field and green house against targeted bugs even commercialized on a big range in many of the realm (Kranthi *et al.*, 2005). The production and protection mechanism of cotton needs to know the variation of season and time for prolific productivity. The toxic protein must be in proper amount which is applied to cotton to provide stability to the cotton plant and for protecting from harmful insect pests. But many researchers of in Australia, USA, India and China have concluded that the amount of *Bt* proteins in cotton changes with the season and the effect of lepidopteron pests also fluctuate in the production of *Bt* cotton (Greenplate *et al.* 2001; Mahon *et al.*, 2002).

The age of the plants also creates variations in the production of *Cry* gene. The *Cry* gene production decreases as the age of the plant increases. To conclude the outcome of endotoxin depends on the age of the plant that young cotton plant production is higher than the aged plant. As a result, the effect of harmful insects' pests also increases with the age of GM plant increase. The different parts of the plant like leaves, bolls, square i.e. carry different effects of the insecticide α-endotoxin as the age increases especially after flowering (Greenplate *et al.*, 1998).

Abiotic stress like nitrogen deficiency (Coviella *et al.*, 2002; Greenplate *et al.*, 1998), high temperature (Chen *et al.*, 2005), high level of CO_2 (Coviella *et al.*, 2000), salinity, water logging (Barrett-Lennard, 2003) and drought (Benedict *et al.*, 1996) helps in facilitating the production of genetically transformed cotton and the appearance of *Cry1Ac* gene in cotton plant increases as well. Plant secondary metabolites is another abiotic factor which is also helpful in the production of *Bt* protein (Kranthi *et al.*, 2005).

A complete awareness of using genetic management or agronomic is essential for dealing with the complex mechanism of *Bt* gene as to control its production with the variations occurs in time, environment and age of the plant. The grower having enough

knowledge of genetic management can gain a large scale of production spending less money (Luo *et al.*, 2008).

Almost all the agricultural system in the world depends on different kinds of agrochemicals which also badly effects the environment causing many types of diseases. A lot amount of money is spending to control the effect of insect on the plant to promote the productivity but the planned action also create hazard for health. Under developed countries like Pakistan has spent 66 million US Dollars in the year 2006-07 (Economic Survey of Pakistan 2008-09) to combat with the harmful insect pest. However, many research institutions have been made to study the situation to find an alternative way to handle the situation (Bakhsh *et al.* 2009).

δ-endotoxins also called crystalline proteins are found in a gram positive, spore - forming, soil-dwelling bacterium that produces *Bacillus thuringiensis* (*Bt*) proteins. *In Bt* the crystal proteins have more than 100 number of sequence encoding *Cry* proteins (Schnepf *et al.* 1998). A large number of harmful insects like *lepidoptera, coleoptera* and *dipteral* are controlled by applying these crystal proteins to the larvae. Therefore, the usage of these Crystal proteins is very high (Gasser and Fraley, 1992). The conventional system of breeding is reliable for achieving the goal in pyramiding the foreign *Bt* gene with traditional protecting skills in a single genetic environment (Altman *et al.* 1996 and Sachs *et al.* 1998).

The major achievement of biotechnology is the introduction of the genetic transformation of genes to the plants which have no demand in the market become very necessary element of daily use and enhancing the capacity of plant to resist harmful insect pest of different kind without rotting (Lycett and Grierson, 1990; Dhaliwal *et al.* 1998). The best achievement is the *Bacillus thuringiensis*(*Bt*) toxin which is best antiseptic. It also contains crystal protein which is toxic to most of the harmful larvae and pests like *Lepidopterans* (Höfte and Whiteley, 1989; Cohen *et al.* 2000), *Coleopterans* (Krieg *et al.* 1983; Herrnstadt *et al.* 1986) and Dipteran insects (Andrews *et al.* 1987). These crystal proteins are safe for human consumptions (BANR, 2000).

The crystal proteins are also used in a large scale to resist harmful insect for the production and safety of rice. When these production enhancers are applied to the plant it appears *Bt* genes like the 35S CaMV promoter (Cheng *et al.* 1998; Alam *et al.* 1999), the

ubiquitin promoter (Chen *et al.* 2005; Tang *et al.* 2006) and the actin facilitator (Wu *et al.* 1997; Tu *et al.*2004). Almost all the crops need CaMV35S enhancer (Odell *et al.*1985) (or nearby facilitator from same viruses) to enhance the transformed genes. It has now been cleared from various studies that the crystal proteins used to resist the effect of many harmful insects is not as same as its performance showed in indoor tests like in laboratory experiences because crystal proteins' performance is different according to the difference in environment and age like factors (Sunilkumar *et al.* 2002).

1.1 AIMS AND OBJECTIVES

The aims and objectives of the study are as follows;

- Gene expression profiling in Pakistani cotton *Bt* genotypes.
- Detection of the types of *Bt* proteins expressing in each plant genotype.
- Confirmation of *Bt* genes in plant genotypes at molecular level.
- Quantification of *Bt* (*Cry1Ac*) gene expression in transgenic *Bt* cotton.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Wu *et al.*, (1997) expressed that the genetics of elite cotton crop was modified by introducing appearance vector having *Cry1Ac* gene and proteinase inhibiting protein *API-B* into it by *Agrobacterium tumefaciens*. Isolation and inheritance of the *Bt* protein was changed via Southern blotting, *Heliothis armigera and* PCR detection according to the Kantrex resistant trial and pest bioassay results, in which some transformants were as per Mendelian examples of legacy, yet others were non-Mendelian examples.

Cohen *et al.*, (2000) stated that there is dependably a hazard that bugs could get to be impervious to *Bt* poison after delayed and rehashed field exposures. "The most down to earth way to deal with drag out the adequacy of *Bt* yields has been refugia methodology and pyramiding of at least two qualities in a similar cultivar.

Greenplate *et al.*, (2001) performed research center studies to portray the *lepidopteran* poisonous quality of cotton cultivar communicating two diverse poison genes from *Bt*, *Cry1A* just, *Cry2Ab* just, or both poisons. In all cases, the *Cry2Ab* segment was the bigger patron to aggregate danger in the two-poison isoline. Poison particular, ELISA tests affirmed that the levels of every poison in tissues of the two-poison isoline were factually not the same as the levels found in the relating tissues of the separate single-poison isoline. Considering the added substance collaboration of poisons, a moderately basic creepy crawly resistance observing system was proposed for the checking of business cotton assortments communicating both poisons.

Adamczyk *et al.*, (2004) expressed that the measure of *Cry1Ac* δ-endotoxin in hybrid Berliner (*Bt*) or Bollgard cotton fluctuates between business cultivars. These distinctions in appearance have been related with existence levels in Lepidoptera, demonstrating that all Bollgard types don't give a similar level of control. The goal of the research was to figure out whether contrasts in general expression among business cultivars of Bollgard cotton were under basic hereditary control. These discoveries could impact the way reproducers select cultivars by assessing for viability in creepy crawly control not withstanding agronomic qualities. Two arrangements of crosses were made in the nursery with cultivars expressing the endotoxin at high as well as low levels. The guardians and F1 and F2 eras were implanted in the fields. The measure of *Cry1Ac* was evaluated utilizing a business ELISA unit. Differences inside the two F2 rearing

populaces were exceedingly substantial in light of hereditary isolation for *Cry1Ac* appearance. Utilizing the altered Castle-Wright equation, the valuation of the quantity of expressing proteins was found little mutually. This evidence demonstrate that hereditary foundation majorly affects *Cry1Ac* expression. Since backcrossing is the essential technique utilized by business cotton reproducers, the determination and utilization of benefactor or potentially intermittent guardians that will bring about an abnormal state of *Cry1Ac* expression is significant.

Kranthi *et al.,* (2005) reasoned that the component of activity of the *Bt Cry* genes includes solubilization of the precious crystal in the pest midgut, when gulped by bugs, toxicant genes tie to particular receptors in the midgut part, and toxicant authoritative in vulnerable bugs disturbs midgut epithelium, along these lines bringing on general dangerous impacts and at last bringing about death of the bugs.

Bakhsh *et al.,* (2009) expressed that the hybrid type communicating *Bt* (*Bacillus thuringiensis*) toxicant is as of now developed on a substantial business scale in numerous nations, however perceptions have demonstrated that it carries on dynamically in toxicant adequacy in contrast to selective bugs underneath ground environments. Comprehension of the worldly and spatial variety in viability and the subsequent technique is basic for cotton safety and manufacture.

Manjunatha *et al.,* (2009) concentrated on four transgenic cotton breeds (RASI XL-708, MRC-7201, SP-11 and MRC-6918,) for *Cry1Ac* gene profiling in completely exposed mortal leaf, initial mortal precandle feather and initial mortal bolls at 80-90, 110-115, 135-140 and 150-165 days afterward of propagating. The outcomes demonstrated that the statement of *Cry1Ac* was declined over season free of the genetic constitution. And between the transgenic plants the appearance level was found distinctive.

Adamczyk *et al.,* (2009) built up a technique to figure out whether contrasts in the general level of *Cry1Ac* between Bollgard colonies could be connected to mRNA transcriptions utilizing quantitative real-time polymerase chain response (qPCR). The business cultivars of Bollgard cotton-type, *Gossypium hirsutum L.*, utilized as a part of the research varied in the measure of communicated *Cry1Ac* genes. He originated that *Cry1Ac* mRNA transcript varies between Bollgard colonies and are connected with

comparing *Cry1Ac* gene levels. The plant-component for which this occurs is still obscure.

Addison *et al.*, (2010) determined that *Cry2Ab* gene level stays very high as compared to *Cry1Ac*, is not influenced by least temperatures down to 2.6°C. As per their discoveries *Cry2Ab* gene level is lessened by least temperatures (edge, ≈14°C) for up to 6 days after every chill except chill has zero effect on *Cry1Ac* gene level.

Zhang *et al.*, (2012) outlined their investigation by utilizing transgenic type cultivars from US and china to analyzed the impacts of higher temperature (37°C) with difference in moisture (half, 70% and 90%) on the transgenic gene expression. As indicated by them at 37°C, temperature and moistness had no consequences for transgenic gene expression at pinnacle squaring point. They additionally broke down that in contrast with the control (25-30°C and 60%-70% moistness), 37°C and 50% dampness diminished leaf transgenic gene ingredients 2.6% to 3.0% at pinnacle blossoming stage and 3.3% to 5.8% at pinnacle boll development point.

Iqbal *et al.*, (2013) relatively calculated the appearance of *Cry1Ac* protein in domestic cotton genotypes underneath ordinary and salt influenced soil in a glasshouse research. As indicated by their discoveries the neighborhood cotton genotypes demonstrated noteworthy contrasts in *Bt* poison level under various salt anxiety levels. They finished up by their investigation that *Bt* poison level reductions with increment in saltiness levels.

Chen *et al.*, (2013) investigated the effect of higher (37°C) and lower (18°C) temperature handling for the time of 24h as well as 48h on the *Cry1Ac* toxicant ingredients of transgenic-cotton at pinnacle boll point. As per them the leaf toxicant levels and their recuperation rate were affected by the span of exciting temperature. According to their glasshouse research, they reasoned that the toxicant detergents of transgenic type recuperated after a brief span (24h) underneath exciting temperature stretch (whichever lower or higher temperature) and the pesticide adequacy recouped totally after higher temperature push (48h) however it was probably not going to recuperate the particular level subsequently long period at lower temperature stretch.

Huang *et al.*, (2014) analyzed the factors of *Bt* protein appearance subsequently economical examinations and came about that assured ranch administrations and specific

utilizations of potassium and phosphate manures has extensive beneficial outcomes on transgenic protein appearance in rancher's fields. Rather than the past work, they described that nitrogen compost has no huge effect on transgenic protein appearance in rancher's fields.

Ullah *et al.*, (2014) concentrated on expression profiling of domestically reared transgenic *Bt*-types via ELISA and were tried for their adequacy in contrast to survival of *Helicoverpa armigera* in switch environment. The populace flow of five sucking arthropods; whitefly, jassid, aphid, thrips and parasites, along the developing period was additionally checked and corresponded with various meteorological elements. Appearance levels of pesticide gene *Cry1Ab/c* was detected to fluctuate fundamentally ($P<0.05$) between types and crosswise over various examining dates. The most elevated average appearance was noted in GN-31 and Sitara-008 and the least in FH-113 and MG-6 though crosswise over inspecting dates the most elevated average appearance was noted at 30 days subsequently rise (DAE) which diminished along the period with least average at 120 DAE. A vital appearance level of *Cry1Ab*/c in leaves was detected at 770 ± 25 ngg-1, for 95% regulator of the objective bugs. Sucking insect populace was observed to be inconstant between both types and the examining dates. A constructive relationship was originated among rain, dampness and sucking arthropods populace in examined cotton fields.

Zaman *et al.*, (2015) in the same way as other cotton developing nations, region underneath the development of GM cotton holding *Cry1Ac* protein has been expanded massively in Pakistan on account of its additional favorable position towards giving imperviousness to bollworms particularly *Helicoverpa armigera*. It has been accounted for that this protein got from *Bacillus thuringiensis* has no negative effect on the loam environment. Research casing biosafety feature of transgene including *Bt* protein are non-existent in our environmental framework. Each nation has diverse microbiota which is the convincing component to configuration research for evaluating the negative effect, assuming somewhat, in our homegrown surroundings. The density of *Cry1Ac* protein in leaf tissues have indicated expanding pattern from 60 DAS to 90 DAS, and reduced at 120 DAS. Comparative outcomes were seen throughout respectively harvesting period. Disparity in gene density was discovered non-substantial over various harvesting years

yet it was important at various testing phases. The *Cry1Ac* toxicant in the loam was short low (78 times) after comparing with leaf. Tests identified with the colony farming units (CFUs) of culturable microscopic organisms, *actinomycetes* and parasites were attempted in the rhizosphere of transgenic and non-transgenic plants at different development phases at 30 days' duration from sowing till development for continuous three-years (2006-2008). A transgenic-cotton type IR-NIBGE-901 (holding *Cry1Ac* protein, reared at NIBGE) and its non-transgenic isogenic type (FH-901) were harvested in NIBGE cotton-field, Faisalabad Pakistan. The density of *Cry1Ac* gene discharged in the loam through root exudates, and in the mortal leaves was quantified 60, 90 and 120 DAS. Comes about demonstrated no critical effect ($P<0.05$) on CFUs of fungus, bacteria and *actinomycetes* among the transgenic and non-transgenic types rhizosphere amid trimming season at one specific phase. Although, time-based and spatial distinctions for microorganisms were seen substantial between the diverse time period (30, 60, 90, 120, 150 and 180 DAS) and the year of development. Subsequently, this research has exhibited that the repetitive development of transgenic-cotton had no substantial effect on soil wellbeing of Pakistan.

Kumari and Kumar, 2015 tested development of *Bt*+ genotypes has abstained from pesticide sprays and consequently corn and cotton ranchers have tremendously profited around the world. Late recordings of hereditarily different *Cry*-resistance in insects of *Bt*+ cotton and corn farms have elevated severe concerns. Inquisitively, main part of *Cry*-resistant pink bollworms originated in specific *Bt*+ cotton fields in India demonstrated homozygous for various connected changes. Moreover, overwhelmingly inheritable *Cry*-resistance and cross resistance among various *Cry*-genes have additionally been distinguished. To stem advancement of resistance in contrast to anti insect gene-toxicants, novel nematology examines on IPM methods, correlative to shelter and *Cry*-stacking advances are unavoidably required.

Sakthi *et al.*, (2015) *Bt*-cotton plants showing a new *cry2AX1* protein comprising of sequences from the *cry2Aa* and *cry2Ac* proteins, determined by the CaMV35S or the EnCaMV35S agents, were created via Agrobacterium-interceded change. Between 40 free PCR-positive trails, 14 delivered *Cry2AX1* gene at perceptible levels. *Cry2AX1* levels in T-plants went from 0.012 to 1.068 Î¼g/g new leaf tissue. Pest bioassays on *Cry2AX1*-showing T-transgenic colonies demonstrated 27.0 to 88.8% mortality of

Helicoverpa armigera. Southern crossbreeding examination exposed single-locus consolidation of the *cry2AX1* protein in two T-plants which brought about 87.5 and 88.8% mortality in *H. armigera*. The occurrence and appearance of the *cry2AX1* protein were affirmed in T-offspring utilizing PCR and quantitative ELISA. The most elevated appearance level of *Cry2AX1* gene noted in the T-group was 0.766 Î¼g/g new leaf tissue, far lower-than appearance of *Cry2Ab* gene (23 Î¼g/g leaf tissue) described in the commercialized *Bt*-cotton, Bollgard II. Disregarding the low *Cry2AX1* level, the mortality in contradiction of *H. armigera* ran somewhere around 33.3 and 85.7% in the T-group. These outcomes demonstrate that this new *cry2AX1* protein delivered an intense insecticidal gene that was viable in contradiction of *H. armigera* in *Bt*-cotton plants at lower density.

2.1 Adaptation of *Bt* Cotton in Pakistan

PARC, (2007) Regarding *Bt* by comparing Pakistan with other countries, it looks a slow and delayed legal adoption of the technology, and lot of benefits. On the other hand, researchers have made efforts and experiments by importing *Bt* seeds himself and practicing on an area of 80% with 36 unapproved verities and by applying Monsanto *Cry1AC* gene event (Mon 531) in all of the verities which is not pretended in Pakistan. These insecticidal verities (IR-CIM-443, TR-CIM-448, IR-NIBGE-2 and IR-FH-901) have been grown on an area of 8000 acres around 2005-2006.

Khuda Baksh, (2009) performed an experiment in which economic performance of *Bt* cottonseed and also traditional *Bt* cotton were analyzed in Punjab. According to socio-economic figures, educated farmers were cultivating *Bt* cotton on large area of their own land and as well as availing an area of rented land. Farmer that fully adopted the *Bt* cotton variety were occupying 44% of the total irrigated land. Around 2005-2006 many other farmers were concerning to adopt transgenic *Bt* cottonseeds. After the awareness of *Bt* seeds, with the ratio of 85%: 100% respectively fully adopters and many other farmers willing decided to adopt and showed keen interest regarding minimum risk of insects. According to the reviews of 67% of farmers- cultivating *Bt* as well as Non- *Bt* seeds, *Bt* seeds were expensive by comparing Non- *Bt* seeds, while according 60% real *Bt* farmers, Non- *Bt* seeds costs friendly as compared to other. In study area many other verities like *Bt*-703 and *Bt*-333 were also cultivated but commonly grown verity was *Bt*-121.

According to the reviews of *Bt* cultivators, *Bt* provides much higher yield and also financial benefits.

2.2 Performance of *Bt* Cotton in Pakistan

Rao, (2009) estimated the activity of 4-*Bt* verities cultivated on an area of 8000 acres in Bahawalpur, Karor Pakka, Muzaffargarh and Multan around 2005-2006. According to review of farmers that adopted *Bt* cotton, different reviews were came to know regarding resistivity and susceptibility to insects by *Bt* cultivators. In the survey report about 65% to 85% distinctions were observed while combination was no more than 2% *Bt* and non *Bt* varieties were mutually effected by aphids, Thrips, whitefly and Jassid on large scale and on the other hand of attics bollworms, Lashkari sundi and American sundi were much less according to the earlier year reports. Non *Bt* cotton was effected by heat stress while on *Bt* cotton, there was no effect of heat stress and cotton leaf curl virus found (PARC, 2007).

Ali *et al.*, (2010) conducted a survey research in which Punjab's 11 districts and 10 Sindh's districts were examined during 2007-2008. Two models were collected from 126 sites to perform immune-strip analyzing for the detection of *Cry* genes from the sample of Punjab's districts was 90% (76/84) while from the district of Sindh 81% (34/42) *Bt* genes were observed. According to the result, *Cry1Ac* protein was found positive while the *Cry2ac* and *Cry1F* proteins were found negative. The status of *Bt* cotton was rechecked by a survey in the following year and the outcome of the survey was much satisfactory because in all those provinces *Bt* cotton was practicing on large scale. This study was the first scientific study of the usage of the illegal *Bt* cotton throughout the country.

2.3 Spatio-Temporal Expression of Insecticidal Genes

Manjunatha *et al.*, (2009) sewed *Bt* seeds and examined four different transgenic *Bt* cotton (MRC-7201, MRC-6918, RASI XL-708) for *Cry1Ac* gene profiling in completely exposed terminal leaf, after 80-90, 110-115, 135-140 and 150-165 days consequently and found 1st terminal bolls. And this it is shown that different appearance levels were seen in the selected transgenic verities and with the passage of time the appearance of *Cry1Ac* was decreased and has no relation with the genotype. There also appeared densely Toxin leaves along with squares and bolls.

2.4 Factors affecting to Endotoxins

Genetic makeup can be changed by a wide range of factors like alteration in segment of nucleic acid, promoter's class, hybrid DNA's set, inner cellular climate/ environment and many exterior atmospheric agencies, including number of Hybrid DNA, the inserting points of the cassette.

Jang *et al.*, (2009) established an experiment in which using ultrafiltration, protein absorption detersive ion Toxin simplification were determined. Lipopolysaccharides (LPS) in detached state wield harmful impression that occupied in the outer most membrane of gram negative bacteria. LPS has 3 important contributions like Lipid A, which is a harmful character, O-specific polysaccharide and a core specific polysaccharide. And 10-30 kDa is an approximate molecular mass of LPS monomer among vesicles and monomers form under physiological circumstances LPS lives in equal proportion 100kda ultra-filtration was found more effective (97% LPS removal) to remove LPS along with low proteins density (2.0mg/ml) rather than higher density (20.1mg/ml) while miner proteins density in the existence of various detergents as Tween 20 (0.5%), Triton X-100 (1.0%) and taurode-oxycholate was found more effective to remove when comparing by higher protein density.

Abid *et al.*, (2011) conducted a research to find out the impact of soil effecting the Transgenic *Bt* cotton (crops) output collection of soil samples was performed from 3-districts like Multan Rahim Yar Khan and Mianwali. A function known as Cobb Douglas Production function was used to measure. The effect of agronomical and demographical factors on the Transgenic *Bt* cotton. There were some variables which were significantly and positively in a very proper manner played their role towards better transgenic *Bt* production as ground composition costs, seed costs, fertilizer costs, labor costs and binary variable of soil type by which the after effects regarding assay/anatomy measured. On the other hand, some complimentary but non-prominent factors were irrigation and spray costs.

2.5 Impact of *Bt*-Toxin

Viktorov, (2008) thoroughly studied the effect of specie on the genetically changed plant by drawing results from different experiments and also studied their pleiotropic effects as well as their interaction with biota soil. He came to know that some

experimental techniques lack sufficient test items and also the used methods are insufficient to meet the current needs. Therefore, it is difficult to achieve the planned targets. By applying transformed Bt plants in huge amount shown that it has got negative bad impacts on the environment. Many examples have proved this impact, for example where ever these genetically changed plants are cultivated, there stored a large amount of *Bt* toxin in soil. And to decompose these genetically changed plants, a long time is needed. Finally, the biologically action of science on these transformed plants is very little as compared to the controlled plants. "Pleiotropy" a phenomenon that is created by the addition of δ-endo toxin, which is encoded by gram positive spore making bacterium B, this activity has proved from a profoundly study of ten years on industrial implementation of genetically transformed plants. Lastly, there is a number of evidence which proves that different agricultural pest family have become able to fight against the *Bt* toxin, which resulted wasting of genetically transformed plants.

Naranjo, (2009) conducted a research in Arizona to determine the effect of Transgenic *Bt* cotton on three main insects. Sprayed and sprayed *Bt* cotton and non-*Bt* cotton were used to observe the predator-prey rate for *Lygus hesperus* knight and *Bemisva tabacci*. *Bt* cotton ever get any related possible effects from predators although by knowing chosen Predators feast activity. Similar ratio of preying determined in *non-Bt* and sprayed *Bt* cotton types. According to sentival eggs and pupae of pectinophora gossypiella whereas *Bt* type has unrelated. The interdependency during the ratio of the predation formation. *Bt*-cotton revealed modest reduction in circumstances of various predation taxa and functions against natural odds.

Badea *et al.*, (2010), cultivated genetically changed corn having event MON-810 which were encoding the *Cry1Ab* protein in enrolled environment and in three different kind of soils. At the final stage of growing season the plants were then integrated into the soil. Using the ELISA assay during vegetable growth and afterward of incorporation of biomass different soil kinds were taken for finding and measuring the quality of *Cry1Ac* gene in leaves, seeds and roots. *Bt*-gene capacity was noticed ass low aged tissues and seeds and the transformed plants grown in different types of soil shown no difference. After the vaccination of plant biomass, the content of *Bt*-gene was high but in elapse of 3-4 months the quantity of *Bt*-gene was decreased. it was concluded that the integration

of cotton into three different kind of soil, *Cry1Ab* gene was not procured. And during the cultivating season variation of appearance of MON-810 was found in both roots as well as in leaves.

Khan *et al.*, (2010), studied some other types of genetically transformed cotton including (*Bt*-3701, *Bt*-W1, *Bt*-1524, *Bt*-496, *Bt*-703, *Bt*-802, *Bt*-313, *Bt*-333, *Bt*-121 and *Bt*-196) and found their action against *Bemisia tabaci* (Whitefly) in 2008-2009. He thus became able to know that different genetically engineered cotton behaves differently like the *Bt*-496 shown popular unsafety against *B. tabaci* (4.52 population/leaf), while *Bt*-3701 proved to be safe against the *B-tabaci* (3.19 population/leaf) and *Bt*-121 (3.55 population/leaf) has a normal insecurity against the *B. tabaci* in 2008. And the other genotype mentioned above, compared to Bt-3701, *Bt*-496, and *Bt*-121shown same effect with the population ranging from 1.97 to 2.75 on single leaf. *Bt*-496 shown different safety measure when studied in the year 2009. It shown safety against Bt-333 (4.25 population/leaf) and maximum against *B. tabaci* (4.97 population/leaf). And other varieties proved to be equivalent to the population range of (2.09 to 3.59/leaf) except *Bt*-703, which shown large resistance against the *B. tabaci* (1.41 population/leaf). After this study, he observed four various leaves physically and structurally. These features contain concentration of the hair, heaviness and length of Lamina and also include glands of gossypol and these glands and heaviness of Lamina depicted positive correlate with population/leaf of *B. tabaci* when studied in the year 2008. And approaches in 2009 expressed that length of hair and gossypol glands have negative relation while, sunshine has a positive effect on *B. tabaci* population.

Thomazoni *et al.*, (2010) bent upon the influence of *Bt* cultivar Nuopal in contrast to non-*Bt* cultivar Delta Opal on mark, unmark and congenital plague via laying on two cross sectioning ways under usual growth estate. Normal figure of mark plagues creatures for the one as well as the other cross sectioning ways was very less in transgenic type. A very big distinction was exposed between transgenic and non-transgenic type for the centric tree cross sectioning way in the diverseness of non-mark plague defined by Shannon-Wiener catalogue, as via this cross sectioning way no distinction was turn up for congenitally happened opponent.

Rao *et al.*, (2011), when CIM-482 and PHYB protein were transmuted to confirm the appearance by PCR. It was detected that 75 pc enhanced in RLGPR (relative growth rate), (SWR) stem weight ratio raised to 52 pc and the degree of fresh and dry 6 mass fuel was increased by 70 pc after maturation in 10 days. Thus the outcome proved that genetically transformed seeds have large capability of production as compared to the non-genetically transformed plants as the reason is slow chlorophyll breakdown and also be well matches those places having relatively long growing time of year. The developed cotton plant's physiology is effective in enlarging the size of fruit as well as enhances the number of fruit is because of the eminent rate of growth. It can also be concluded that the number of bolls increase in phytochrome B-plants resulting lowering of height in contrast to the treated cotton plant.

Rawat *et at.*, (2011) conducted a research in which impacts of *Cry1Ac* were determined. According to the research, *Cry1Ac* out growth had the damaging developmental and growth impacts in vivo as well as in vitro. Various types of *Cry1Ac* proteins were used to develop *Bt* plants and many experiments were conducted but in number of plants the *Cry1Ac* protein was not expressed even with the presence of marker protein npt11, *Cry1Ac* protein was found absent during southern blot analysis. And those plants in which *Cry1Ac* protein was detected, their phenotype was aberrant badly. *Cry1Ac* protein showed inhibition of in-vitro regeneration. When this protein was taken to transform in hybrid tobacco. *Cry1Ac* protein was found positive in just 21 self-dependent plants of the tobacco, while the remaining of 145 were found negative. So it indicates that *Cry1Ac* gene is required in large amount for the transformation. But gene formulation can be enhanced by preying the gene in to chloroplast.

Ying *et al.*, (2011) experimented to determine the digestion and detoxification of proteins activeness as well as nutritious constitution towards unlike time interruptions within the quarter arthropode's (Instar) army worms enriched upon transgenic cotton along with non-transgenic cotton. In both nutritious constitution and particular protein activeness. There was found substantial deviations in between Instar army worms enriched with Trypsin as well as entire superoxide dismutase (T.SOD) activeness that was enriched upon Transgenic type, on other hand lipase, acetyl cholinesterase and carboxylase activeness was found in low quantity rather than the non-transgenic cotton

enriched warms since whole enriching time (1,6 and 24 hour). Inside all cotton collection bunch, signification distinctions were examined amongst enrichment periods in *S-exigua* enriched on transgenic for one day and night, reflected extremely down released acidulous and whole amino acidulous than with in that enrichment up to sixty minutes. Just as the low actions of lipase and trypsin were able to see in warmly enrichment on transgenic cotton for one day and night t than that for sixty minutes in *S.exigua* enrichment on transgenic cotton for one day and night actions of carboxylesterase and acetylcholine-esterase were majorly higher than that for sixty,240 and 260 minutes. The answers show that, the mutual action among cottons collection and enrichment time majorly influenced the actions of those enzymes *S.exigua*. For this reason, below the situations choice stress of poisoned protein examining the secular apportion and neutralize enzyme actions in the mass of S. exigua in reaction against *B-thringenesis* may deliver a fruitful estimate of the transforming patience of the insects feeding on plants.

Yu *et al.,* (2011) conducted a survey on an already conducted research between 2005 to 2010 to observe positive impacts on predators, (Larva endurance, body mass and usance) in which *Bt* sensitive worm as prey had been used. Any negative impact could not be detected while using *Bt*-sensitive or as well as infected herbivore as feed. According to the conclusion, transgenic cultivars has not any lineal impact on NTOs although having contract array of acti-vessels. Transgenic cotton is being used enormously progressively showing outstanding results regarding labor/farmer health natural impacts and as well as economic profit. Transgenic cultivars are the helpful portion of integrated pest management system against the insects that infect the crops on large scale.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was carried out at International Islamic University Islamabad (IIUI) in collaboration with National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Center (NARC), Islamabad during the year 2016.

3.1 Plant Material

Seventy-two local *Bt* cotton genotypes were analyzed in the present study to evaluate the expression of *Cry1Ac* gene in under glass house conditions. The seeds of these genotypes (NCVT-1601 to NCVT-1672) were provided by National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad.

3.1.1 Pot Experiment and Growth Condition

The plant material was sown in glasshouse (Biosafety Level-II) at (NIGAB), where initially temperature was maintained between 25-30°C. Five seeds were sown in each pot (38cm in diameter and 40cm in length), filled with 15kg of prepared soil and plants were irrigated on need basis.

3.2 Soil Preparation and Temperature Treatment

3.2.1 Soil Preparation

The soil was prepared before filling in the pots. Mixture of soil, farm yard manure and sand in the ratio of 2:1:1 was fully ground and sieved. Each pot was prepared by the same ratio of mixture (soil, farm yard manure and sand).

3.2.2 Temperature Treatment

All the genotypes were cultivated under normal temperature condition (25-30°C) in glasshouse. Out of 72 genotypes, ten genotypes with high expression of *Bt* toxin were selected and grown in three replicates under same conditions (25-30°C). The temperature was regularly observed in different containments/glasshouses.

Fig. 3.1 Local Cotton Genotypes in Glass House.

3.3 DNA Extraction

DNA was extracted from fresh leaf of each entry with Cetyl Trimethyl Ammonium Bromide (CTAB) method following Doyle and Doyle (1990). 2-3 leaf samples were taken and grinded in mortar and pestle with liquid nitrogen. The crushed sample was taken in eppendorf tube and 900 µl of 2% CTAB having 0.5% β-mercaptoethanol was added. The eppendorf tubes were incubated at 65°C for 30 minutes. Chloroform: Isoamylalcohol (24:1) solution of 0.8 volumes (720 µl) was added to all the samples. Then sample was centrifuged at 14000rpm for 10 minutes. After centrifugation, supernatant was transferred to fresh tubes and equal volume of chilled Iso-propanol was added. Samples were incubated at 20°C for 20 minutes. The sample was centrifuged at 14000rpm for 20 minutes. The supernatant was discarded; the pellet was washed with 70% ethanol and dried. The pellet was dissolved in 100µl ddH_2O. The product was analyzed on 1.5% agarose gel.

3.4 DNA Quantification

The DNA quantity and quality was done by Nano-drop TM (IMPLEN Serial No. 1404 UK) at 260 and 280 nm. The ratio 260/280 was used to access the contamination with protein and RNA. From the stock, samples were diluted to working concentration of 50ng/µl with the following equation.

$$C_1V_1 = C_2V_2$$

3.5 Polymerase Chain Reaction (PCR)

For confirmation of transgene at DNA level, gene specific (Mon531) event primers pair were used for the amplification of *Cry1Ac* gene in transgenic cotton genotypes.

Polymerase Chain Reaction (PCR) was carried out using Veriti® thermal cycler (Applied Biosystems, USA) following the method described by Yang et al. (2005).

Table 3.1: Set of primer used in Polymerase Chain Reaction (PCR).

Target	Primer Name	Sequences (5′ ------ 3′)	Specificity	Amplicon size (bp)
Mon-531	Mon-531 F	AAGAGAAACCCCAATCATAAAA	Genome/*Cry1Ac*	346bp
	Mon-531 R	GAGAATGCGGTAAAGATACGTC		
Mon1445	Mon1445 F	AGGCATCTTGAACGATAGCCTTTC	--------	599bp
	Mon1445 R	AACACCTAATACAAGTCATACATACA		
Mon 15985	Mon 15985 F	ATTTGATGCACTATGTCTTCGTCTA	--------	175bp
endogenous	Mon 15985 R	CAATGGAATCCGAGGAGGT		
Cotton	Sad1 F	CCAAAGGAGGTGCCTGTTCA	Stearoyl-ACP	107bp
endogenous	Sad1 R	TTGAGGTGAGTCAGAATGTTGTTC	desaturase	

The thermal cycler was programmed as follows;

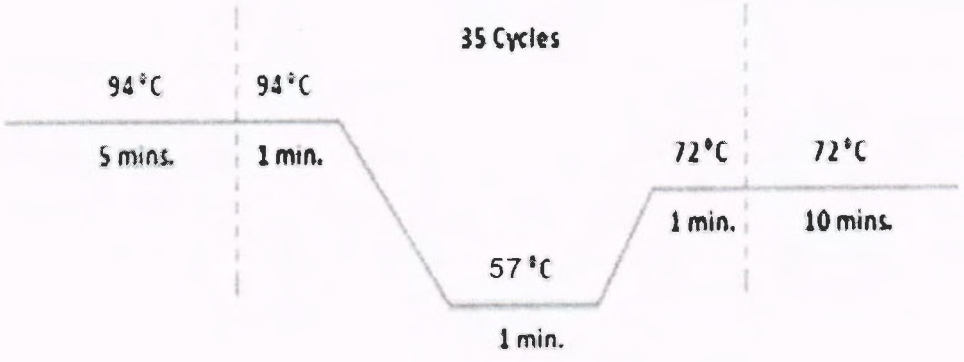

Figure 3.2: Thermo cycler programming for PCR

3.6 Horizontal Gel Electrophoresis

Amplified products were separated on 0.5µg/ml ethidium bromide containing 1.5% agarose gel in 1X TBE (10 mMTris-Borate, 1 mM EDTA) buffer at 100 V for 30 min. After electrophoresis amplified products were visualized at 260nm wavelength on trans-illuminator and photographed by a gel documentation system. Amplicon size of *Cry1Ac* gene was confirmed with 100 bp ladder.

3.7 Immuno-Strips Assay

Approximately 100mg fresh leaf tissue of each genotype was collected for Immuno-Strips assay. Samples were prepared as per manufacturer's instructions (Agdia Inc. USA) and were checked for the detection of *Cry* protein(s) *Cry1Ac*, *Cry2A* band *Cry1F*. Strips marked as "SAMPLE" were carefully inserted into micro tube with prepared extract to avoid their entrance more than 0.5cm during the reaction time. The appearance of control line with in 3 minutes during reaction was considered as valid. Two types of immunostrips (Cat. No: STX010300 and Cat. No: STX06800) were used in assay. Reaction results were recorded as positive (+) and negative (-) on the bases of test line appearance on the strip within due time.

Fig. 3.3 ImmunoStrip Analysis of Cotton Genotypes

3.8 Sandwich-ELISA

After 80 days of sowing, quantification of *Cry1Ac* encoded toxin was carried out by sandwich-ELISA. Third fully expanding leave tissues of each plant were used for analysis. Twenty milligram (20mg) fresh plant tissues were sampled, grinded manually by using extraction buffer provided by manufacturer of kit (Envirologix Inc. USA). At room temperature plant tissues samples (20mg) were crushed in 500 ml extraction buffer, the grinded samples were centrifuged for 3-4 minutes at 10,000-12,000 rpm. In 900µl dilution buffer, 100µl of sample supernatant was diluted and mixed gently. For the detection of unknown protein (*Cry1Ac* toxin) in the given sample, four calibrators (standards) were used i.e. control, 1.5, 10 and 25ppb (provided by manufacturer). Calibrators were used to draw the regression equation based on their final optical density. For measuring the unknown expression of the protein (*Bt* toxin), 100µl of each standard was put into their respective wells. Following the calibrators, 100 µl of each sample (diluted sample) was pipetted into the rest of the wells. The ELISA kit plate was shaken for 30 seconds. After shaking, 100µl enzyme conjugate (provided by manufacturer) was added into each well. The kit plate was shaken again for 30 seconds. After shaking, the

kit was covered and kept as such for 1 hour at room temperature. After incubation period, material of the wells was removed gently and by washing buffer the wells were washed. Washing was repeated for 3-5 times. The wells were dried well by inverting on dry tissue papers for several minutes. After drying, 100µl substrate was added into each well. Kit was shaken again for 30 seconds after covering, following 30 min of incubation at room temperature; color of wells was changed to blue. Then, by addition of 100µl stop solution color of the well was changed from blue to orange. Finally, to calculate toxin level, sample optical density was measured by Microplate Reader (BIO-RADiMarkTM) at wavelength of 450nm.

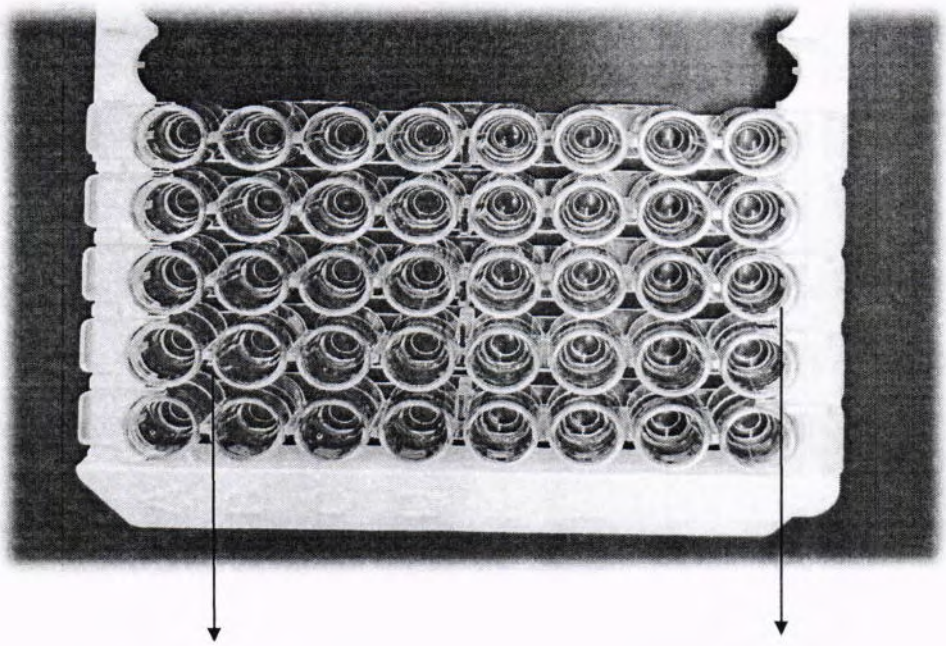

Fig. 3.4: Quantification of *Bt* toxin *Cry1Ac* by Sandwich ELISA

3.9 Statistical Analysis

Simple regression analysis was carried out to calculate toxin levels as µg/g in different plant genotypes using Microsoft Excel software.

RESULTS

4. RESULTS

4.1 Immunostrip assay for *Bt* genes detection

Seventy two (72) cotton genotypes were tested by immunostrip assay for the detection of *Bt* genes viz., *Cry1Ac*, *Cry2Ab* and *Cry1F*. The results for immunostrip assay are shown in the Figure 4.1. The control line representing the validity of reaction and the remaining two lines are showing the positive reaction for *Cry1Ac* and *Cry2A/1F*. Results showed that all the genotypes carried *Cry1Ac* gene except for NCVT-1653, NCVT-1663 and NCVT-1665. All the genotypes showed 100% negative reactions by immunostrip assay for *Cry2Ab* (Bollgard-II event) and *Cry1F* (Wide Strike event) genes.

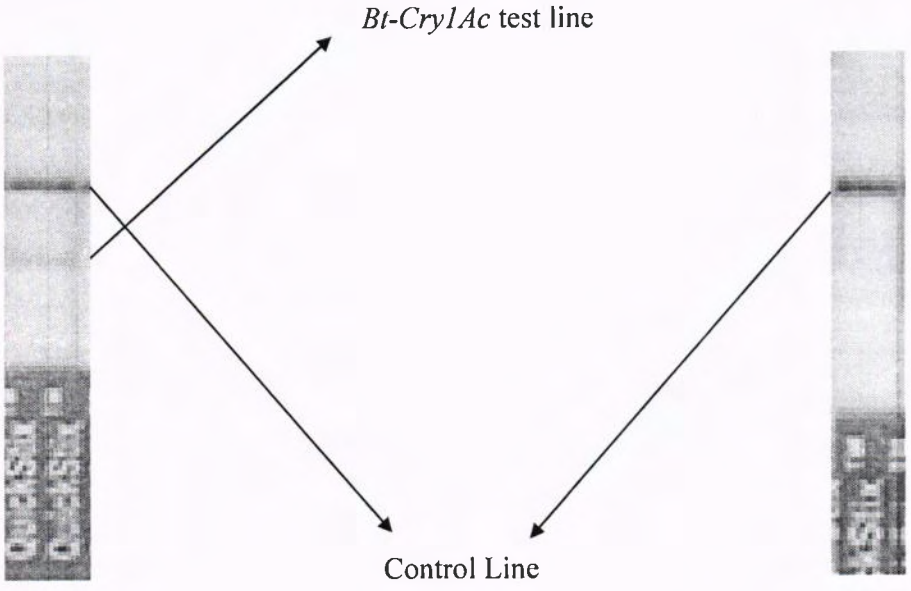

Fig. 4.1 (a) ImmunoStrip showing positive result

Fig. 4.1 (b) ImmunoStrip showing negative result

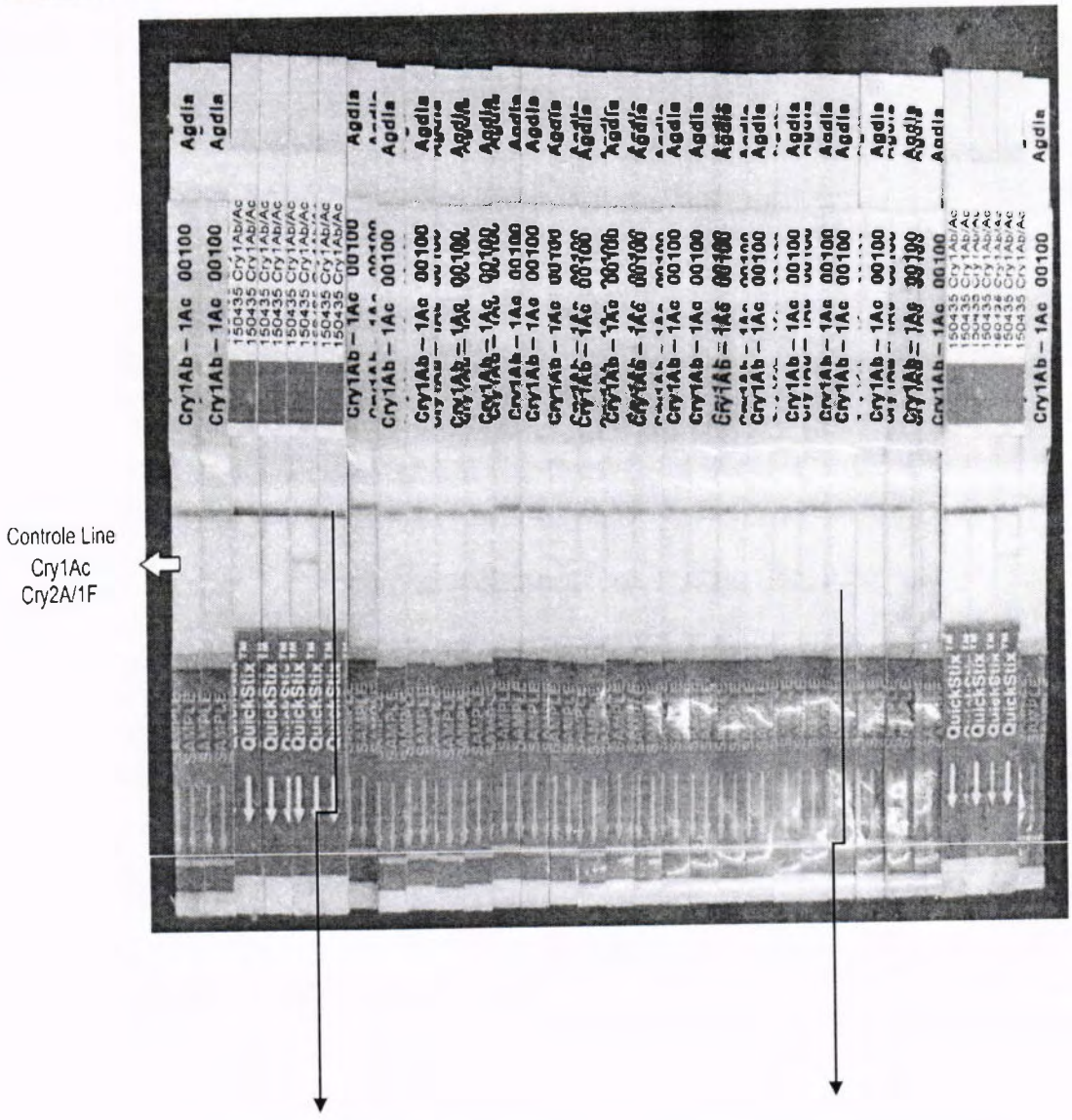

Figure 4.2: Immunostrip analysis for *Bt* toxin detection of cotton genotypes

Table 4.1: Results for immunostrip assay of 72 cotton genotypes.

Entry	Cry1Ac	Cry1Ab/1F	Entry	Cry1Ac	Cry1Ab/1F
NCVT-1601	YES	No	NCVT-1637	YES	No
NCVT-1602	YES	No	NCVT-1638	YES	No
NCVT-1603	YES	No	NCVT-1639	YES	No
NCVT-1604	YES	No	NCVT-1640	YES	No
NCVT-1605	YES	No	NCVT-1641	YES	No
NCVT-1606	YES	No	NCVT-1642	YES	No
NCVT-1607	YES	No	NCVT-1643	YES	No
NCVT-1608	YES	No	NCVT-1644	YES	No
NCVT-1609	YES	No	NCVT-1645	YES	No
NCVT-1610	YES	No	NCVT-1646	No	No
NCVT-1611	YES	No	NCVT-1647	YES	No
NCVT-1612	YES	No	NCVT-1648	YES	No
NCVT-1613	YES	No	NCVT-1649	YES	No
NCVT-1614	YES	No	NCVT-1650	YES	No
NCVT-1615	YES	No	NCVT-1651	YES	No
NCVT-1616	YES	No	NCVT-1652	YES	No
NCVT-1617	YES	No	NCVT-1653	YES	No
NCVT-1618	YES	No	NCVT-1654	YES	No
NCVT-1619	YES	No	NCVT-1655	YES	No
NCVT-1620	YES	No	NCVT-1656	YES	No
NCVT-1621	YES	No	NCVT-1657	YES	No
NCVT-1622	YES	No	NCVT-1658	No	No
NCVT-1623	YES	No	NCVT-1659	YES	No
NCVT-1624	YES	No	NCVT-1660	YES	No
NCVT-1625	YES	No	NCVT-1661	YES	No
NCVT-1626	YES	No	NCVT-1662	YES	No
NCVT-1627	YES	No	NCVT-1663	No	No
NCVT-1628	YES	No	NCVT-1664	YES	No
NCVT-1629	YES	No	NCVT-1665	YES	No
NCVT-1630	YES	No	NCVT-1666	No	No
NCVT-1631	YES	No	NCVT-1667	YES	No
NCVT-1632	YES	No	NCVT-1668	YES	No
NCVT-1633	YES	No	NCVT-1669	YES	No
NCVT-1634	YES	No	NCVT-1670	YES	No
NCVT-1635	YES	No	NCVT-1671	YES	No
NCVT-1636	YES	No	NCVT-1672	YES	No

YES = *Bt* gene presence, No = *Bt* gene absence

Results for immunostrip assay are shown in the Table 4.1. 'YES' is for the presence and 'No' is for the absence of *Bt* gene.

4.2 PCR analysis

This test was designed to ascertain the ownership of transgene event(s) at DNA level. The event in each genetic engineered background is always unique and cannot be modified through back crossing the plant material. Three plants per entry were analyzed for this test.

The overall results of each tested genotype are shown in the table 4.1. Through PCR, all of the entries/genotypes gave positive results/amplifications by using primer sets for Mon-531 with the product size of 346 bp (figure 4.3).

All the genotypes showed negative results for Mon15985 event specific for Bollard-II® except in control with amplicon size of 175 bp (Line NCVT-1517 from NCVT 2015 used as positive control). The construct information of Bolgard-II® revealed that reporter GUS gene has been ligated to *Cry2A*. So to further verify and cross check the result, PCR was conducted to detect the presence of GUS gene which could not show the presence of GUS in any genotypes except in control with amplicon size of 498bp, thereby, confirming the absence of Mon15985 (Bolgar-II) in the all of entries submitted for NCVT-2016.

Also not even a single genotype gave positive results for Mon1445 (Roundup Ready) in PCR test except control with amplicon size of 599 bp (NCVT-1553 from NCVT-2015 used as positive control). (Figure 4.4,4.5 and 4.6).

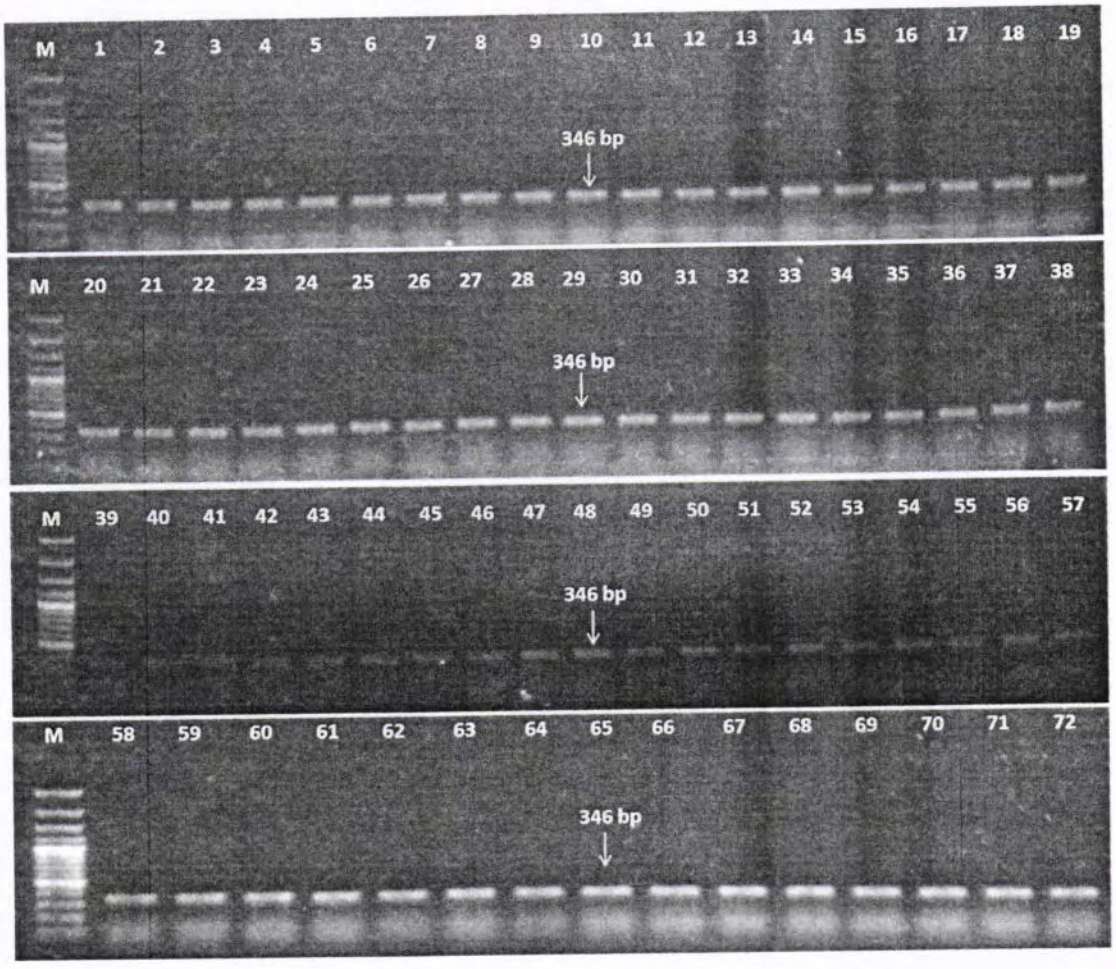

Figure 4.3: Amplification of Mon-531 event (*Cry1Ac*) in 72 genotypes through PCR: NCVT-1601 to NCVT-1672 is the amplification of Mon-531 (346 bp). 100 bp (Left) Ladder is used.

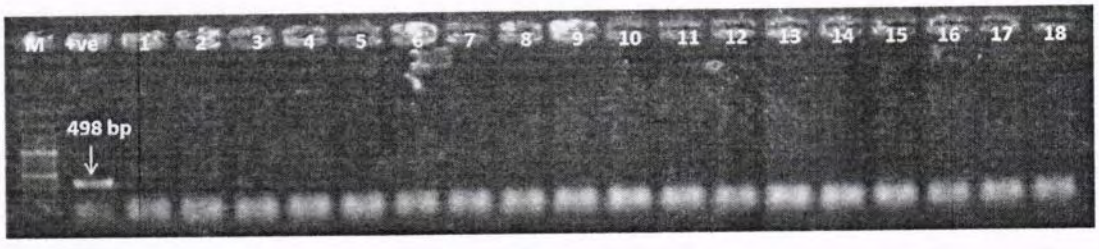

Figure 4.4: Amplification of Uid3A (GUS) gene (indicating *Cry2Ab* according to construct information) in NCVT genotypes through PCR: No amplification was shown in any genotype with Uid3A gene (GUS) Primers (498 bp) except in positive control. 100 bp (Left) ladder used.

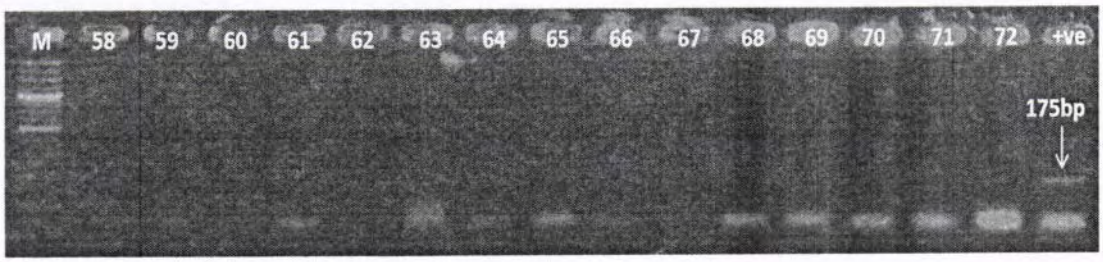

Figure 4.5: Amplification of Mon-15985 event (*Cry2Ab*) in NCVT genotypes through PCR: No amplification was shown in any genotype with Mon-15985 event except in Positive control (175 bp). 100 bp (Left) ladder is used.

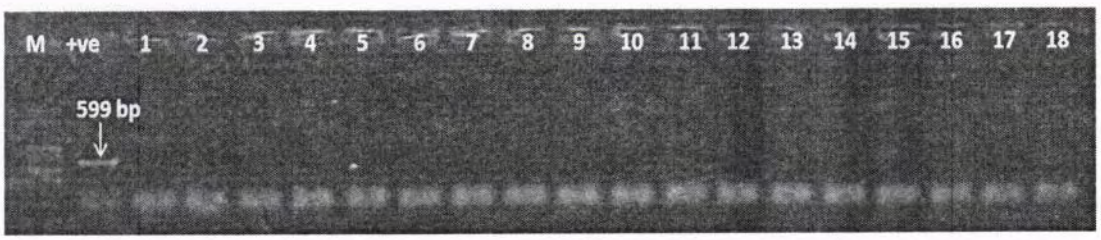

Figure 4.6: Amplification of Mon-1445 event (CP-4, Roundup Ready™) in NCVT genotypes through PCR: No amplification was shown in any genotype with Mon-1445 event except in Positive control (599 bp). 100 bp (Left) ladder used.

4.3 Enzyme Linked Immunosorbent Analysis (ELISA)

4.3.1 Quantification of *Cry1Ac* toxin in leaf tissues at 80 DAS (days after sowing).

The purpose of this test was to measure the level of toxin production. This is extremely important to avoid resistance build up in the targeted insect pests (Bollworms) and to establish expression profiles of cotton lines being tested in national trail. Literature has reported a level of 1.5-1.9 µg/g fresh leaf weight (Kranthi *et al.* 2005) for durable resistance, thus the level of toxin gains vital importance.

Sandwich ELISA was performed additionally to quantify *Cry1Ac Bt* toxin after the collection of positive results for transgenic *Cry1Ac* genotype by performing immustrrip assay. The results for all the genotypes are shown in Table 4.2 and Figure 4.7. It is obvious from results that the quantity of *Cry1Ac* toxins in leaf tissues on fresh weight basis of all control plants under normal temperature condition 25-30°C ranged from 0.0145 – 5.6074µg/g. Four genotypes (NCVT-1646, NCVT-1658, NCVT-1663 and NCVT-1666) gave 0.00µg/g level of *Bt* toxin.

Table 4.2: Quantification of *Cry1Ac* toxin (µg/g) level for all (NCVT-1601 – NCVT-1672) genotypes at 80*DAS. 0.0000 – 5.6074

Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin
NCVT-1601	2.6689	NCVT-1625	0.0547	NCVT-1649	1.9575
NCVT-1602	1.9826	NCVT-1626	0.0462	NCVT-1650	4.0460
NCVT-1603	2.2099	NCVT-1627	0.0607	NCVT-1651	1.2170
NCVT-1604	2.6903	NCVT-1628	0.0308	NCVT-1652	1.6694
NCVT-1605	1.9196	NCVT-1629	0.0342	NCVT-1653	2.9871
NCVT-1606	0.4609	NCVT-1630	1.9603	NCVT-1654	0.9597
NCVT-1607	0.8316	NCVT-1631	0.7558	NCVT-1655	3.7258
NCVT-1608	0.0253	NCVT-1632	1.9245	NCVT-1656	2.9862
NCVT-1609	1.2656	NCVT-1633	2.6894	NCVT-1657	1.5230
NCVT-1610	2.4456	NCVT-1634	0.4601	NCVT-1658	0.0000
NCVT-1611	0.0434	NCVT-1635	0.0436	NCVT-1659	5.6074
NCVT-1612	0.3368	NCVT-1636	0.0895	NCVT-1660	1.7166
NCVT-1613	0.3370	NCVT-1637	0.9765	NCVT-1661	5.3202
NCVT-1614	0.7143	NCVT-1638	0.0687	NCVT-1662	4.6184
NCVT-1615	0.4653	NCVT-1639	0.2754	NCVT-1663	0.0000
NCVT-1616	0.0252	NCVT-1640	0.8650	NCVT-1664	3.9601
NCVT-1617	1.8265	NCVT-1641	0.4661	NCVT-1665	0.9098
NCVT-1618	2.3757	NCVT-1642	1.0410	NCVT-1666	0.0000
NCVT-1619	1.5382	NCVT-1643	0.5906	NCVT-1667	1.3813
NCVT-1620	1.1765	NCVT-1644	1.7299	NCVT-1668	1.1489
NCVT-1621	0.9312	NCVT-1645	3.3999	NCVT-1669	1.9339
NCVT-1622	1.4795	NCVT-1646	0.0000	NCVT-1670	0.1105
NCVT-1623	1.5433	NCVT-1647	3.5539	NCVT-1671	0.0145
NCVT-1624	1.5863	NCVT-1648	2.7689	NCVT-1672	4.1008

*DAS (Days after sowing). Tabulated values are the mean of three plants tested for each entry.

NCVT-1646, NCVT-1658, NCVT-1663 and NCVT-1666 are with 0.00µg/g level of *Bt* toxin.

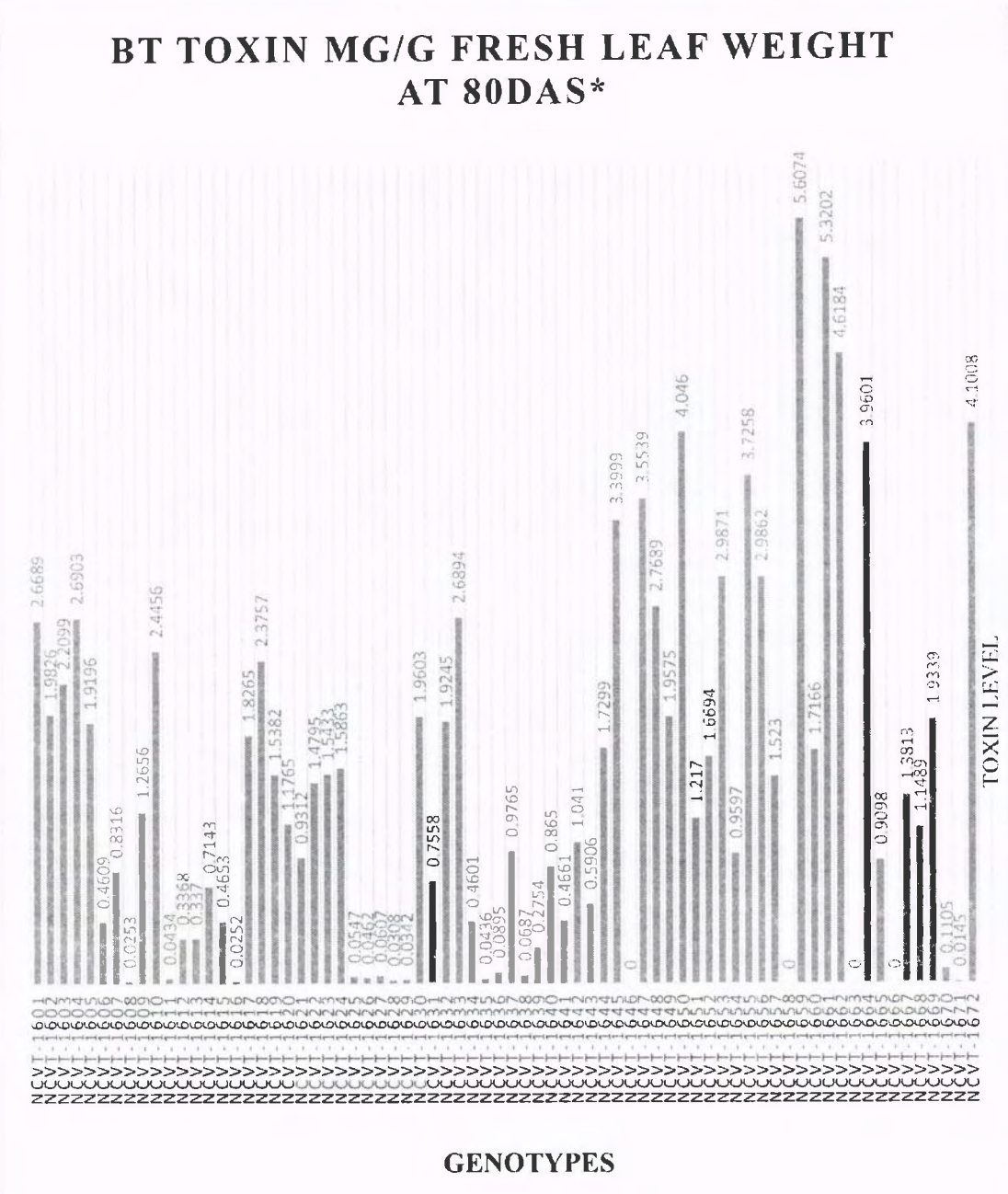

Figure 4.7: *Bt* toxin (*Cry1Ac*) level for all genotypes at 80 *DAS (days after sowing)

Table 4.3: High *Cry1Ac* Toxins (µg/g) level in 18 cotton genotypes. 2.2099 – 5.6074 µg/g

Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin
NCVT-1601	2.6689	NCVT-1645	3.3999	NCVT-1656	2.9862
NCVT-1603	2.2099	NCVT-1647	3.5539	NCVT-1659	5.6074
NCVT-1604	2.6903	NCVT-1648	2.7689	NCVT-1661	5.3202
NCVT-1610	2.4456	NCVT-1650	4.0460	NCVT-1662	4.6184
NCVT-1618	2.3757	NCVT-1653	2.9871	NCVT-1664	3.9601
NCVT-1633	2.6894	NCVT-1655	3.7258	NCVT-1672	4.1008

Eighteen genotypes (NCVT-1601, NCVT-1603, NCVT-1604, NCVT-1610, NCVT-1618, NCVT-1633, NCVT-1645, NCVT-1647, NCVT-1648, NCVT-1650, NCVT-1653, NCVT-1655, NCVT-1656, NCVT-1659, NCVT-1661, NCVT-1662, NCVT-1664 and NCVT-1672) expressed the highest level of *Bt* toxin ranged from 2.2099 – 5.6074µg/g.

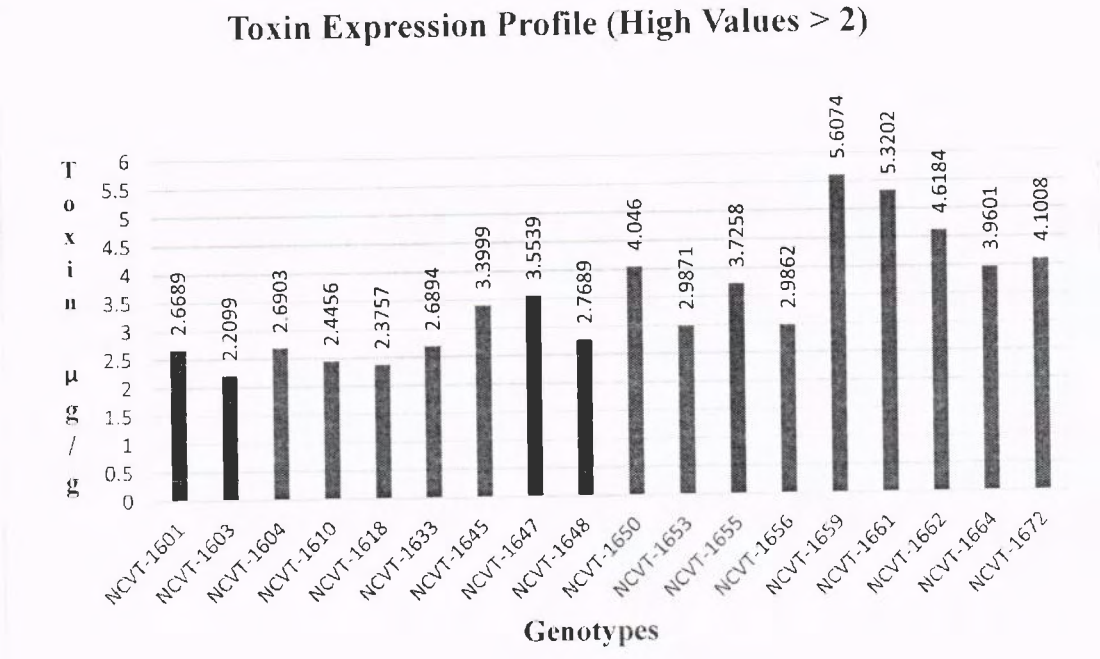

Figure 4.8: High *Cry1Ac* Toxins (µg/g) level in 18 cotton genotypes.

Table 4.4: Medium level of *Cry1Ac* Toxins (µg/g) in 21 cotton genotypes. 1.0410– 1.9826 µg/g

Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin
NCVT-1602	1.9826	NCVT-1623	1.5433	NCVT-1651	1.2170
NCVT-1605	1.9196	NCVT-1624	1.5863	NCVT-1652	1.6694
NCVT-1609	1.2656	NCVT-1630	1.9603	NCVT-1657	1.5230
NCVT-1617	1.8265	NCVT-1632	1.9245	NCVT-1660	1.7166
NCVT-1619	1.5382	NCVT-1642	1.0410	NCVT-1667	1.3813
NCVT-1620	1.1765	NCVT-1644	1.7299	NCVT-1668	1.1489
NCVT-1622	1.4795	NCVT-1649	1.9575	NCVT-1669	1.9339

Twenty-one genotypes (NCVT-1602, NCVT-1605, NCVT-1609, NCVT-1617, NCVT-1619, NCVT-1620, NCVT-1622, NCVT-1623, NCVT-1624, NCVT-1630, NCVT-1632, NCVT-1642, NCVT-1644, NCVT-1649, NCVT-1651, NCVT-1652, NCVT-1657, NCVT-1660, NCVT-1667, NCVT-1668 and NCVT-1669) expressed the medium level of *Bt* toxin ranged from 1.0410– 1.9826µg/g.

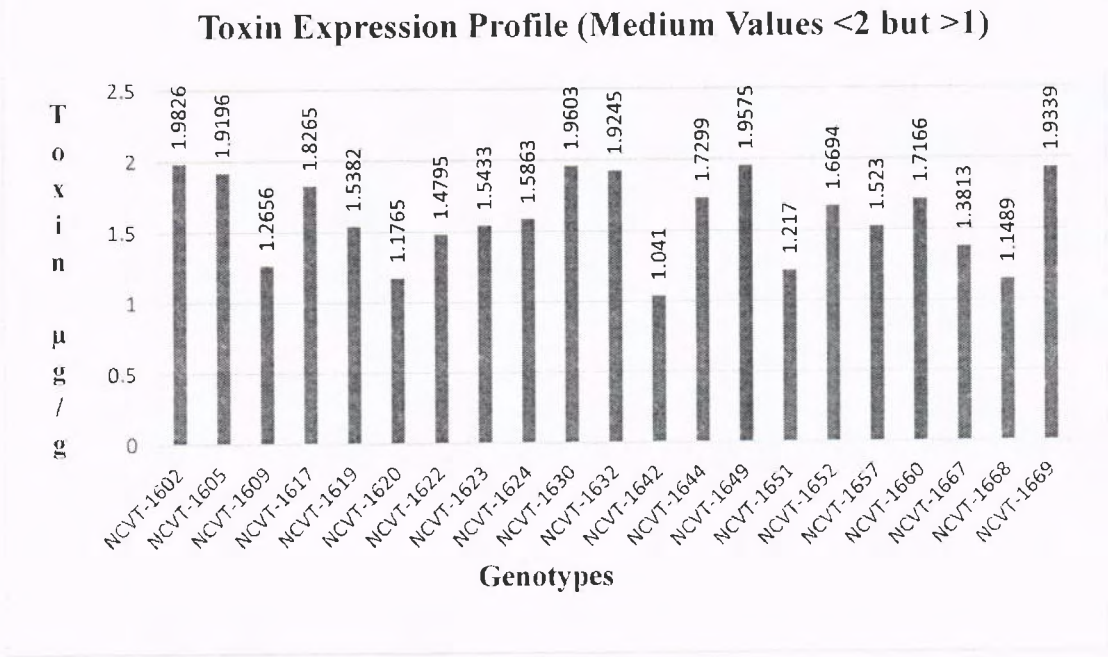

Figure 4.9: Medium level of *Cry1Ac* Toxins (µg/g) in 21 cotton genotypes. 1.0410– 1.9826 µg/g

Table 4.5: Low level of *Cry1Ac* Toxins (µg/g) in 17 cotton genotypes. 0.1605– 0.9765 µg/g.

Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin
NCVT-1606	0.4609	NCVT-1621	0.9312	NCVT-1641	0.4661
NCVT-1607	0.8316	NCVT-1631	0.7558	NCVT-1643	0.5906
NCVT-1612	0.3368	NCVT-1634	0.4601	NCVT-1654	0.9597
NCVT-1613	0.3370	NCVT-1637	0.9765	NCVT-1665	0.9098
NCVT-1614	0.7143	NCVT-1639	0.2754	NCVT-1670	0.1605
NCVT-1615	0.4653	NCVT-1640	0.8650	****	****

Seventeen genotypes (NCVT-1606, NCVT-1607, NCVT-1612, NCVT-1613, NCVT-1614, NCVT-1615, NCVT-1621, NCVT-1631, NCVT-1634, NCVT-1637, NCVT-1639, NCVT-1640, NCVT-1641, NCVT-1643, NCVT-1654, NCVT-1665 and NCVT-1670) expressed low level of *Bt* toxin ranged from 0.1605– 0.9765µg/g.

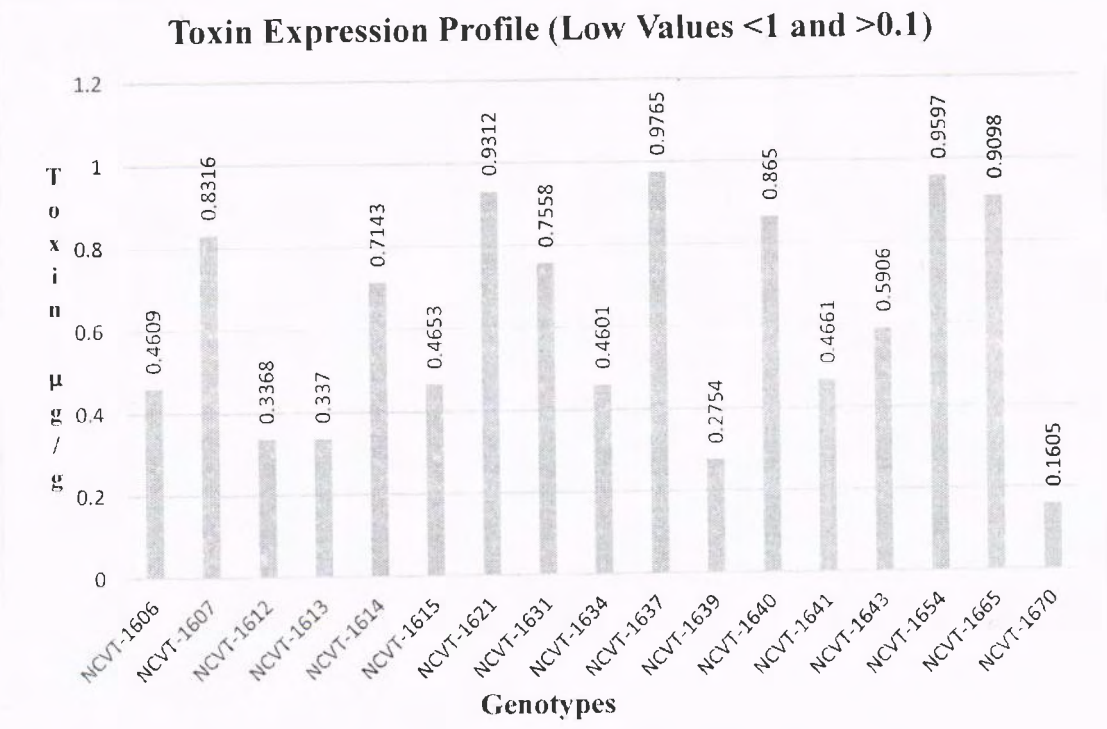

Figure 4.10: Low level of *Cry1Ac* Toxins (µg/g) in 17 cotton genotypes.

Table 4.6: Very low level of *Cry1Ac* Toxins (µg/g) in 12 cotton genotypes. 0.0145–0.0895µg/g

Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin	Genotype	*Cry1Ac* Toxin
NCVT-1608	0.0253	NCVT-1626	0.0462	NCVT-1635	0.0436
NCVT-1611	0.0434	NCVT-1627	0.0607	NCVT-1636	0.0895
NCVT-1616	0.0257	NCVT-1628	0.0308	NCVT-1638	0.0687
NCVT-1625	0.0547	NCVT-1629	0.0342	NCVT-1671	0.0145

Twelve genotypes (NCVT-1608, NCVT-1611, NCVT-1616, NCVT-1625, NCVT-1626, NCVT-1627, NCVT-1628, NCVT-1629, NCVT-1635, NCVT-1636, NCVT-1638 and NCVT-1671) expressed very low level of *Bt* toxin ranged from 0.0145– 0.0895µg/g.

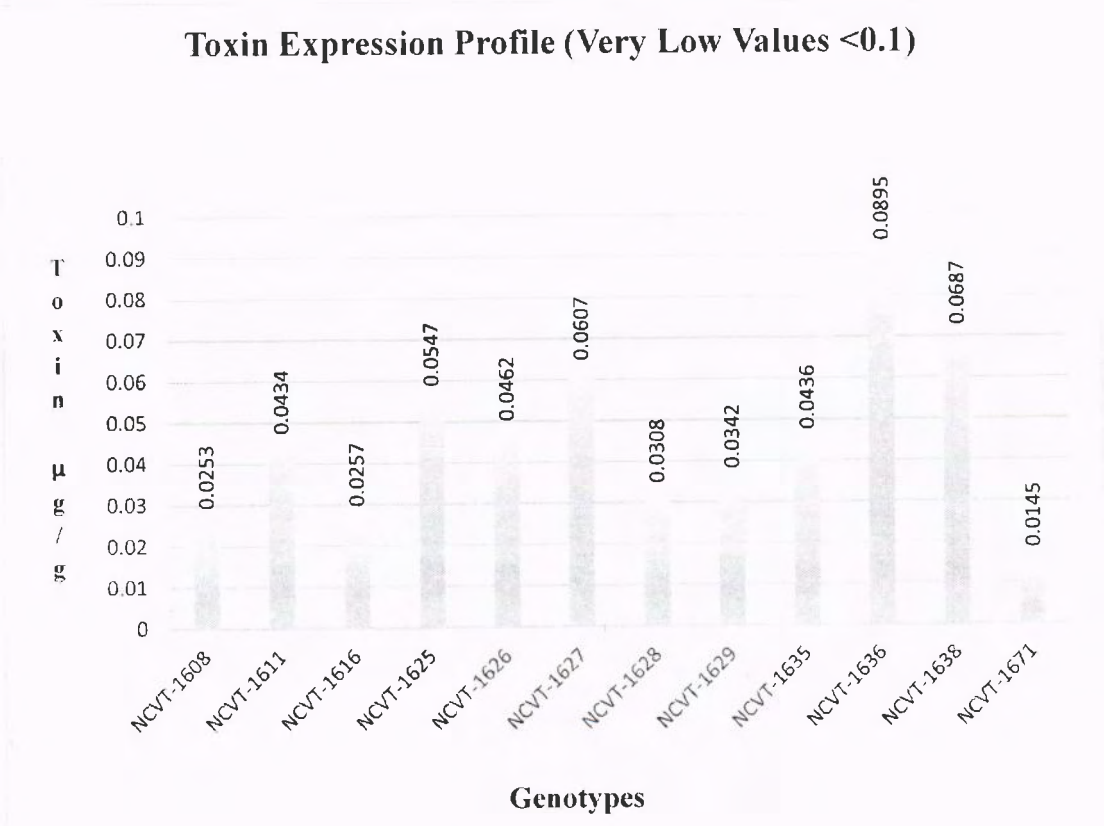

Figure 4.11: Very low level of *Cry1Ac* Toxins (µg/g) in 12 cotton genotypes.

SUMMARY

5. SUMMARY

Cotton is the most popular fiber crop which is used in various commercial consumption specialty it has a vital role in textile industry. It provides many job opportunities and has important role in promoting economy of the country. The cotton textile industry in the past suffered extremely due to some key issues like use of expensive insecticides against the insects in crop which also have environmental hazards. The present study focused on detection of genes expression, which have great resistivity against pests without harming environment. The study of *Cry* genes and transforming these genes into cotton is the important achievement of the biotechnology. The *Cry* gene has ample resistivity against pests and many researchers are busy to enhance the resistivity of these genes. The process of producing enhanced resistive *Bt Cry* genes is very complicated. Researchers used to study the *Bt Cry* gene and continuously throughout the growing season to obtain an international standard environment friendly *Bt Cry* gene (pesticide) threshold level of *Bt* toxin "*Cry*-protein" which is a special type having little harm to the environment and an ample resistance against a verity of pests. Transferring *Cry* genes to cotton not only increase productivity but also help to enlist on legal GM crops adopting countries. Therefor the "*Cry* proteins" has great applications in commercial production. The efficacy of the genotype depends on the level of the *Cry* protein in various tissues of the plant. These are many complications in the appearance of *Cry* proteins which causes the variations. The appearance of the insecticidal protein in the earlier stage of the plant might also be the cause of variation. The insecticidal gene which appears in earlier stage become inactive in later stage. This study is also based on the observations of promoters which enhance the growth of endotoxin regularly during the whole season of the cotton crop. These promoters are also helpful in the expression of transformed *Bt* cotton genotypes. Particularly in the fruiting parts of cotton plant which are susceptible to the insects throughout the growing season. The study of morphological, physiological and molecular behaviors is essential when comparing hybrid over non-transgenic cotton plants. Correlation of level of toxin expression and frequency of insects will be needed for final selection of genotypes for development of local varieties.

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6. REFERENCES

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APPENDICES

7. APPENDICES

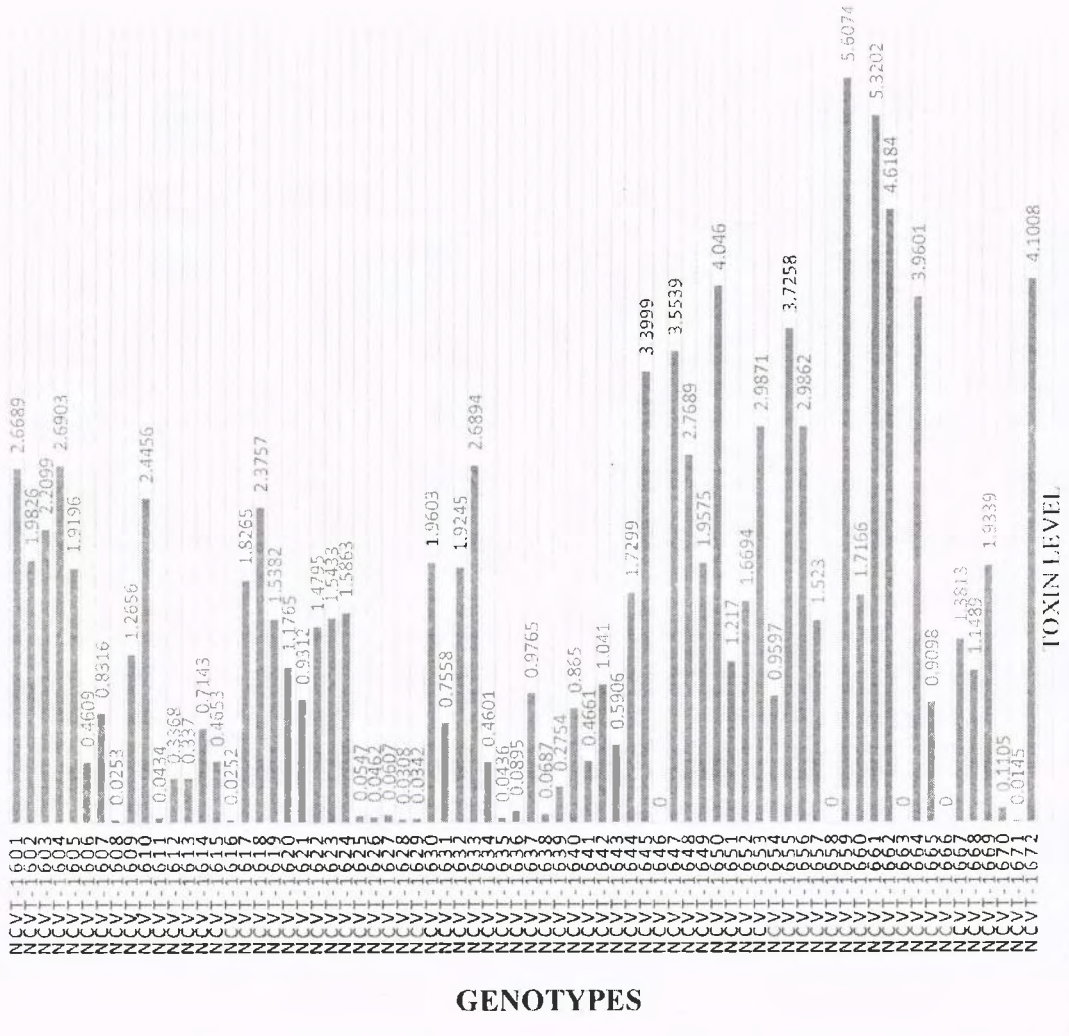

Figure 7.1: *Bt* toxin (*Cry1Ac*) level for all genotypes at 80 *DAS (days after sowing)

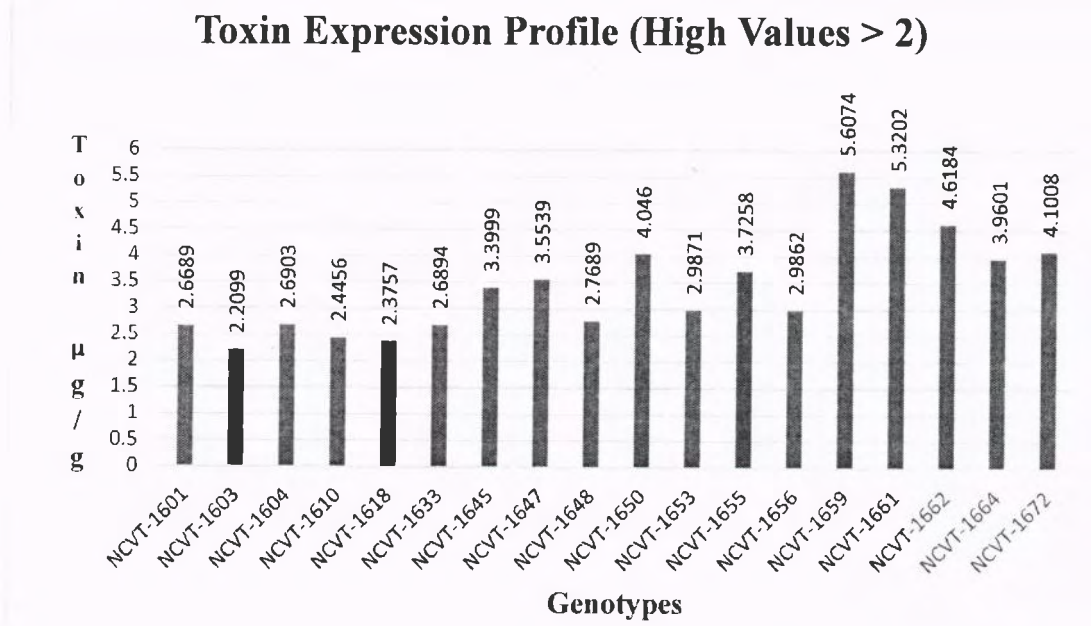

Figure 7.2: High *Cry1Ac* Toxins (µg/g) level in 18 cotton genotypes.

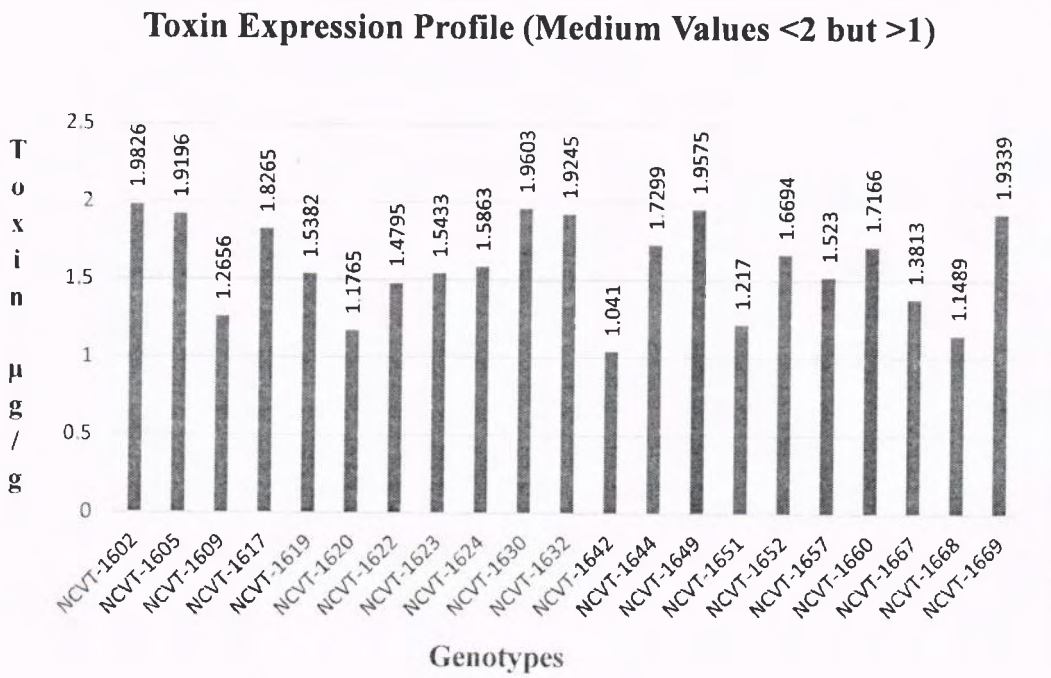

Figure 7.3: Medium level of *Cry1Ac* Toxins (µg/g) in 21 cotton genotypes. 1.0410– 1.9826 µg/g

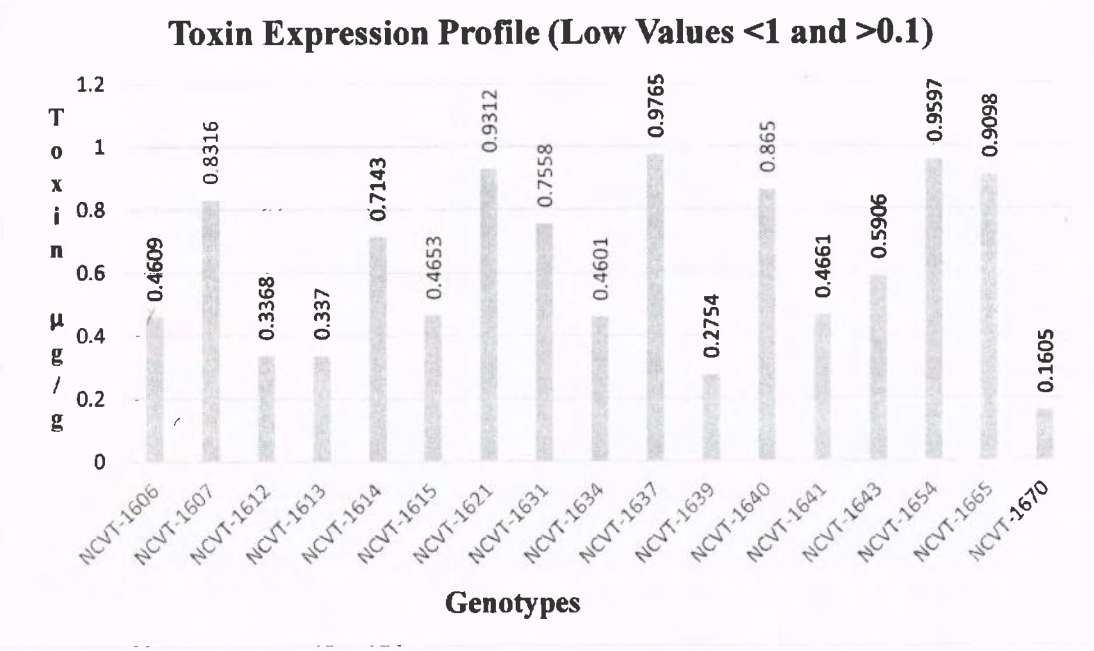

Figure 7.4: Low level of *Cry1Ac* Toxins (µg/g) in 17 cotton genotypes.

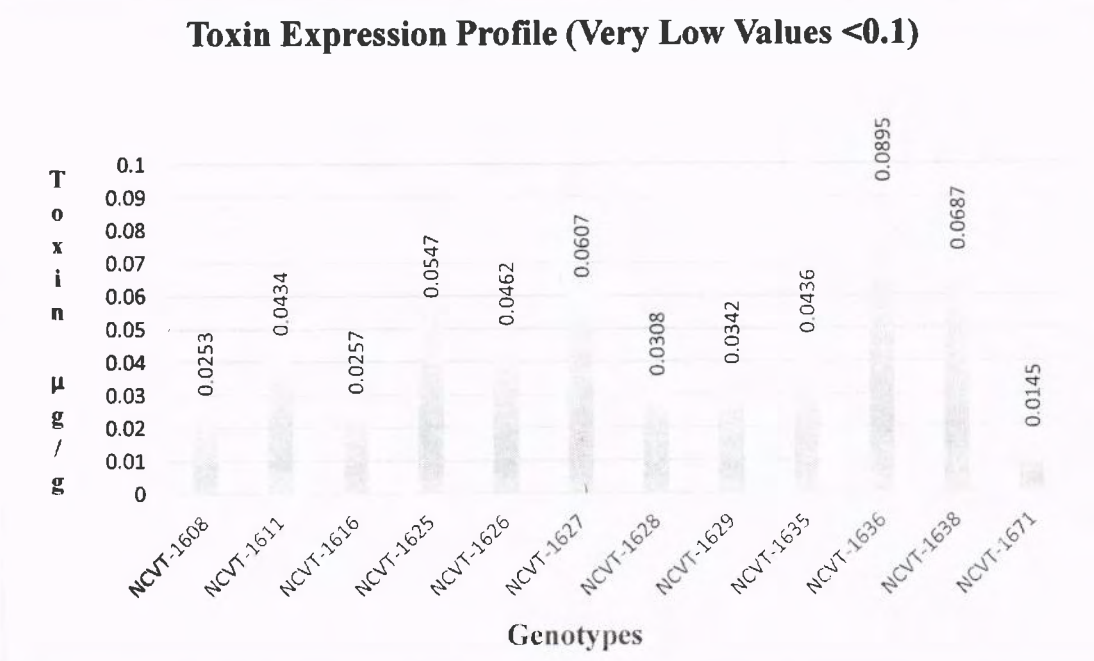

Figure 7.5: Very low level of *Cry1Ac* Toxins (µg/g) in 12 cotton genotypes.