**The Journal of Animal & Plant Sciences, 29(2): 2019, Page: 531-538**
**ISSN: 1018-7081**

# SIGNIFICANCE OF CROSS COMBINATIONS FOR DEVELOPING Bt COTTON VARIETIES FOR OPTIMUM GENE (CRY1AC) EXPRESSION LEVEL REQUIRED FOR BETTER BOLLWORM CONTROL

R. Anjum[1], M. J. Baloch[2], G. M. Baloch[2] and Q. Chachar[3]

Department of Plant Breeding and Genetics, Central Cotton Research Institute, Sakrand, Pakistan; [2]Department of Plant Breeding and Genetics, [3]Department of Crop Physiology, Sindh Agriculture University, Tandojam, Pakistan

Corresponding Author's Email: rajputrehana@yahoo.com

## ABSTRACT

Level of Bt gene expression plays a vital role for better management of cotton bollworm. So the selection of cotton varieties having maximum gene expression at the period of bollworm attack ultimately manage pest better and yield more than the varieties failed to control bollworm attack. Thirty six cross combinations developed from six non-Bt females and six males containing Bt gene (Cry1Ac) were crossed in 6 × 6 North Carolina Design II fashion, were planted during the study period. All the crosses were evaluated to detect the Bt gene by immunostrip assay that exposed the presence of gene Cry1Ac in all crosses. Through quantitative ELISA, Bt gene expression was quantified. Gene expression of Cry1Ac at 30 days after planting in all thirty six cross combinations varied from 1.003 to 2.814µg/g. The top ten cross combinations having maximum gene expression (2.814 to 1.170 µg/g) were selected for further bioassay studies, the mortality% of *Earias* spp in leaves at 60, 90 and 120 days after planting were also recorded. At 60 days after planting (DAP) mortality was recorded 80% while at 120 DAP mortality percentage dropped to unacceptable level and ranged between 33 to 28%. However some combinations proved effective at 120 DAP as well. Our studies proved that selection of combinations can improve pest management and ultimately enhance yield for the betterment of the farmers and for the country as a whole.

**Key words:** Bt Cotton, gene (Cry1Ac) expression, Cross combinations, bollworm.

## INTRODUCTION

Insects pest of cotton are the main cause of low production of cotton in Pakistan. Farmer's rely more on insecticides to manage these pests to get maximum production before genetically modified cotton. After the commercialization of transgenic cotton containing gene Cry1Ac protein from *Bacillus thuringensis,* it became a major component of its integrated insect pest management for minimizing the economic load of major lepidopterous control. Cry1Ac protoxin is a crystal protein produced by the bacterium *Bacillus thuringiensis* (Bt) during sporulation. Cry1Ac is one of the delta endotoxins produced by this bacterium which act as insecticides. Because of this, the genes for these have been introduced into commercially important crops by genetic engineering (e.g. cotton and corn) in order to confer pest resistance on those plants. (Acharjee and Sarmah, 2013; McLean, 2011; Liu *et al*., 2012). Cry1Ac gene also has capacity to control *Heliothis armigera, Earias spp* and *Pectinophora gossypiella* when they feed on a substrate having toxic Bt protein (Crickmore *et al*., 2005; OECD 2007; Qaim, 2009).

In Pakistan single gene Cry1Ac Bt cotton varieties have been grown for more than a decade. In contrary, the cotton farmers in advance countries have a choice for third generation Bt cotton varieties. Resultantly being in use for such a long period, the poor bollworm control is evident in the field and scientists are reporting Bt resistance in bollworms (Naveed *et al.,* 2014). Since the content of δ-endotoxin proteins associates with the efficacy against target insects (Olsen *et al*. 2005; Adamczyk *et al*., 2001), it is imperative to maintain the consistency of expression of Bt genes at adequately high levels to get insect control (Llewellyn *et al*., 1994). Studies undertaken by Rochester, (2006) reported variation in Cry1Ac protein expression among candidate cotton cultivars; he also proved that it is feasible for cotton breeders to lessen the risk of damage from lepidopterous pests by selecting cultivars that exhibit high protein levels.

Studies undertaken by previous researchers (Greenplate *et al*., 2000, Adamczyk and Sumerford, 2001, Holt *et al*., 2002, Gutierrez *et al*., 2006) concluded that the efficacy of Bt cotton against bollworm varies among cotton varieties depending on the expression of Cry1Ac gene and fluctuations throughout the raising season (Olsen *et al*., 2005; Karanthi *et al*., 2005; Bakhsh *et al*., 2010; Adamczyk *et al*., 2009). The production of endotoxin level also decreased with the age of crop (Wan *et al*., 2005). Poongothai *et al.* (2010) observed that the effectiveness of Bt cotton against targeted pests differs with plant age owing to decrease in endotoxin protein quantity in plant tissues. It was also observed boll

damage increases with growing age of plant. Prior to commercialization, it is requisite to quantify the Cry1Ac gene expression level at different plant growth stages for appropriate pest control.

It is evident from the previous studies that the quantity and gene expression level can be enhanced by selecting suitable combiners. So, the primary goals of present research were to find out different cross combinations, to quantify the variation in Cry1Ac protein at various plant growth stages, and to evaluate the level of gene expression required for efficient bollworm control.

## MATERIALS AND METHODS

Research was conducted at Central Cotton Research Institute research farm, Sakrand and in the laboratories of Central Cotton Research Institute, Multan and Sakrand. Six non-Bt female parents viz., CIM-573, CRIS-342, FH-941, Sadori and CRIS-134 were crossed with six Bt male parents viz., FH-113, IR-NIBGE-1524, IR-NIBGE-3701, MG-6, AA-802 and Sitara-008 in North Carolina Design-II fashion during 2011, hence thirty six crosses were developed. The seed of thirty six cross combinations were planted during cotton season 2012 in a randomized complete block design (RCBD) with four replications. Each cross in a replication was sown four rows of 6.0 m length, maintained 30.0 cm distance within plants and 75.0 cm row to row distance. Three consecutive plants from each cross per replication were selected and tagged at 30 days of planting.

**Immunostrip Assay:** For detection of Bt gene (Cry1Ac), around hundred milligram of fresh leaf tissue per cross was collected at 30 days after planting for immunostrip assay. Agida immunostrips were used as per manufacturer's protocol. All the crosses depicted positive response for the presence of gene Cry1Ac.

**Sandwich-Enzyme Linked Immunosorbent Assay (ELISA):** All the crosses were exposed to sandwich ELISA to quantify the Cry1Ac toxic level at 30 days after planting (DAP). Ten out of thirty six crosses with the highest expression level at 30 DAP were selected for further testing of toxic level of Cry1Ac at 60, 90 and 120 DAP. Third leaf from top of each cross was collected for ELISA test following the procedure applied by Chen *et al*. (1997).

**Bioassay of *Earias* spp:**

**1. Field collection and culture maintenance:** More than 350 half to full grown larvae of *Earias spp* were collected from okra crop around Sakrand and were fed on okra fruit in glass jars in the laboratory (25± 1°C). Pupae of *Earias* thus formed were isolated and adults emerged from them were kept in glass jars (3.5 lit capacity). Tissue paper pads soaked with liquid moth diet were provided in plastic petri dishes and lappy liner strips hanged in the jars for oviposition. The jars were covered with nappy liner with the help of rubber bands. Cotton pads soaked with water were put on inner side of glass jar as well as on nappy liner cover to maintain moisture. About 25 moths were kept in a glass jar. Nappy liner and cotton pads having eggs were collected daily and kept in the laboratory in plastic jars (8.5 lit capacity) covered with muslin cloth. Eggs hatched in 3 days, however, fresh and insecticide free okra fruits were provided one day before so that newly hatched larvae could settle on readily available natural food.

**2. Bioassay under laboratory conditions:** Small bolls of age 14-18 days were collected from ten tested crosses at 60, 90, and 120 days of planting were used to bioassay. Five 3-day old larvae of *Earias spp* were released on the crushed small bolls of each crosses individually in plastic petri dishes of 5 cm diameter with same sized filter paper disc underneath to absorb any excessive moisture that could condense in petri dishes. Petri dishes were covered with lid and were sealed with surgical tape to avoid get away of the larvae. The petri dishes were kept in the laboratory (26±1°C) in 5 replicates and larval mortality/survival was recorded at 24-hrs after exposure. Larvae were touched with camel hairbrush and considered alive if movement was observed. Software Statistix 8.1 was used for data analysis for toxin level and bollworm mortality percentage.

## RESULTS

**1. Immunoassay for quantitative estimation of Cry1Ac protein:** Immunostip assay conducted at 30 days after planting confirmed the presence of Cry1Ac gene in all thirty six crosses. However all crosses showed the different level of quantification of Cry1Ac toxin level at 30 days after planting (Table 1). In our studies toxic level of Cry1Ac of thirty six crosses was in the range of 1.003 to 2.841µg/g dry weight in leaves at 30 days after planting. The highest Cry1Ac toxic levels of 2.841, 2.555, 2.441, 2.393, 2.031, 1.386, 1.334, 1.324, 1.278 and 1.170 µg/g were recorded in cross combinations CRIS-134 × MG-6, BH-167 × MG-6, CRIS-134 × IR-NIBGE-1524, FH-941 × IR-NIBGE-1524, CRIS-134 × IR-NIBGE-3701, CIM-573 × IR-NIBGE-1524, CRIS-134 × FH-113, CIM-573 × IR-NIBGE-3701, CRIS-342 ×IR-NIBGE-3701 and BH-167 × AA-802 respectively and these top ten hybrids were selected for further bioassay studies and insect mortality % at 60, 90 and 120 days after planting.

At 60 days after planting, the significant difference in toxic levels of Cry1Ac in upper canopy leaves was recorded in selected ten crosses of having

different parentage combination (F= 44.32; df= 9, 40; P=<0.0001). Maximum gene expression of 1.508µg/g dry weight in upper canopy leaves was observed in cross CRIS-134 × MG-6, followed by 1.334µg/g in CRIS-134 × IR-NIBGE-1524, 1.316 µg/g in CRIS-134 × IR-NIBGE-3701, 1.304 µg/g in FH-941 × IR-NIBGE-1524, 1.265µg/g in BH-167 × MG-6 and 1.230µg/g in CIM-573 × IR-NIBGE-1524 respectively. The rest of the combinations showed less than 1.0µg/g of gene expression in upper canopy leaves (Table 2). Only one hybrid FH-941 × IR-NIBGE-1524 showed 1.067µg/g of gene expression at 90 days after planting and was significantly higher than the most of the cross combinations (F=34.87; df= 9,40; P=<0.0001), followed by 0.949µg/g in CRIS-134 × MG-6, 0.923µg/g in CIM-573 × IR-NIBGE-1524, 0.723µg/g in CRIS-134 × IR-NIBGE-1524, 0.696 µg/g in CIM-573 × IR-NIBGE-3701 and 0.673µg/g in CRIS-134 × IR-NIBGE-3701 respectively. The rest of the hybrids showed less quantity of gene expression (Table 3). At 120 days after planting, all ten crosses quantified less than 1.00 µg/g of gene expression level with maximum 0.893 µg/g in hybrid CRIS-134 × IR-NIBGE-1524 followed by 0.834 µg/g in CIM-573 × IR-NIBGE-1524, 0.826 µg/g in CRIS-134 × MG-6 and 0.824 in BH-167 × MG-6 respectively and significant difference were observed in gene expression levels (F= 43.98; df= 9, 40; P=<0.0001) among crosses (Table 2).

Overall interaction effect between age intervals and crosses were also statistically significant (F= 6.74; df= 18, 120; P=<0.0001). Higher gene expression level of 1.09 µg/g was recorded in cross CRIS-134 × MG-6 followed by FH-941 × IR-NIBGE-1524, CIM-573 × IR-NIBGE- 1524, CRIS-134 × IR-NIBGE-1524, BH-167 × MG-6 showing gene expression of quantity 1.04, 1.00, 0.98 and 0.92 µg/g respectively (Table 2).

**2. Bioassay on immature bolls for *Earias spp*.:** *Earias spp* was selected for present research since it was considered one of the most destructive and harder species tolerating a wider range of climatic conditions corresponding to optimum temperature and humidity in Pakistan. The mortality% of *Earies* spp in bioassays with young bolls at 60, 90 and 120 days after planting is given in Table 3. At 60 days after planting, mortality was recorded 80 % and significantly higher (F= 26.33; df= 9, 40; P=<0.0001) in crosses CRIS-134 × MG-6, BH-167 × MG-6, CRIS-134 × IR-NIBGE-1524 and FH-941 × IR-NIBGE-1524 compared by other crosses respectively (Table 3). While at 90 days after planting, mortality percentage declined and was recorded 60 % in CRIS-134 × MG-6, BH-167 × MG-6, CRIS-134 × IR-NIBGE-1524 and CIM-573 × IR-NIBGE-1524 were significantly higher (F=24.75; df=9, 40; P=<0.0001) compared with other crosses at 90 days after planting. The mortality percentage at 120 days after planting dropped to unacceptable level and ranged between 33 to 28 percentages. However, significantly higher (F=4.24; df= 9, 40; P=< 0.0007) mortality percentage was recorded in crosses CRIS-134 × MG-6, BH-167 × MG-6, FH-941 × IR-NIBGE-1524, CRIS-134 × IR-NIBGE-3701 and CIM-573 × IR-NIBGE-1524 respectively followed by other crosses.

Overall seasonal mean mortality percentage was also significantly higher (18.38; df=18,120; P=<0.0001) in hybrids CRIS-134 × MG-6, BH-167 × MG-6 and CIM-573 × IR-NIBGE-1524 followed by CRIS-134 × IR-NIBGE-1524, CIM-573 × IR-NIBGE-3701, CRIS-134 × FH-113, BH-167 × AA-802, FH-941 × IR-NIBGE-1524, CRIS-134 × IR-NIBGE-3701 and CRIS-342 × IR-NIBGE-3701 respectively. It is quite evident that mortality percentage of *Earias* spp larvae decreases with the passage of time and with the gradual reduction of Bt toxin present in the fruiting bodies (Fig 2).

**Table 1. Immunostrip test and quantification of Cry1Ac toxin Level in 36 $F_1$ hybrids of cotton at 30 days after sowing.**

| $F_1$ Hybrids | Qualitative Immunostrip test (MON 531) | ELISA Cry1Ac (µg/g) 30 days after sowing |
|---|---|---|
| CRIS-134 × MG-6 | +ve | 2.841 |
| BH-167 × MG-6 | +ve | 2.555 |
| CRIS-134 × IR-NIBGE-1524 | +ve | 2.441 |
| FH-941 × IR- NIBGE-1524 | +ve | 2.393 |
| CRIS-134 × IR- NIBGE-3701 | +ve | 2.031 |
| CIM-573 × IR- NIBGE-1524 | +ve | 1.386 |
| CRIS-134 × FH-113 | +ve | 1.334 |
| CIM-573 × IR- NIBGE-3701 | +ve | 1.324 |
| CRIS-342 × IR-NIBGE-3701 | +ve | 1.278 |
| BH-167 × AA-802 | +ve | 1.17 |
| CRIS-342 × MG-6 | +ve | 1.163 |
| CRIS-134 × Sitara-008 | +ve | 1.154 |

| | | |
|---|---|---|
| CRIS-134 × AA-802 | +ve | 1.14 |
| FH-941 × MG-6 | +ve | 1.134 |
| CRIS-342 × FH-113 | +ve | 1.126 |
| FH-941 × IR- NIBGE-3701 | +ve | 1.115 |
| CIM-573 × AA-802 | +ve | 1.087 |
| Sadori × MG-6 | +ve | 1.08 |
| Sadori × IR- NIBGE-3701 | +ve | 1.064 |
| CRIS-342 × IR-NIBGE-1524 | +ve | 1.053 |
| BH-167 × FH-113 | +ve | 1.05 |
| BH-167 ×IR- NIBGE-3701 | +ve | 1.049 |
| Sadori × IR- NIBGE-1524 | +ve | 1.047 |
| CIM-573 × Sitara-008 | +ve | 1.042 |
| Sadori × AA-802 | +ve | 1.039 |
| Sadori × FH-113 | +ve | 1.036 |
| FH-941 × Sitara-008 | +ve | 1.036 |
| FH-941 × AA-802 | +ve | 1.034 |
| BH-167 × IR- NIBGE-1524 | +ve | 1.03 |
| CIM-573 × MG-6 | +ve | 1.027 |
| CRIS-342 × AA-802 | +ve | 1.025 |
| BH-167 × Sitara-008 | +ve | 1.025 |
| CIM-573 × FH-113 | +ve | 1.014 |
| FH-941 × FH-113 | +ve | 1.012 |
| CRIS-342 × Sitara-008 | +ve | 1.003 |
| Sadori × Sitara-008 | +ve | 1.003 |

**Table 2. Cry1Ac expression level (µg/g fresh weight) in upper portion leaves of ten $F_1$ hybrids of cotton at different crop period**

| $F_1$ Hybrids | Toxic level (µg/g) days after planting | | | Mean |
|---|---|---|---|---|
| | 60 | 90 | 120 | |
| CRIS-134 × MG-6 | 1.508 | 0.949 | 0.826 | 1.09 |
| BH-167 × MG-6 | 1.265 | 0.675 | 0.824 | 0.92 |
| CRIS-134 × IR-NIBGE-1524 | 1.334 | 0.723 | 0.893 | 0.98 |
| FH-941 × IR-NIBGE-1524 | 1.304 | 1.067 | 0.739 | 1.04 |
| CRIS-134 × IR-NIBGE-3701 | 1.316 | 0.673 | 0.581 | 0.86 |
| CIM-573 × IR-NIBGE-1524 | 1.230 | 0.923 | 0.834 | 1.00 |
| CIM-573 × IR-NIBGE-3701 | 0.857 | 0.696 | 0.484 | 0.68 |
| CRIS-134 × FH-113 | 0.802 | 0.542 | 0.428 | 0.59 |
| CRIS-342 × IR-NIBGE-3701 | 0.757 | 0.497 | 0.384 | 0.55 |
| BH-167 × AA-802 | 0.536 | 0.207 | 0.158 | 0.30 |

**Table 3. Mortality% of *Earias* in bioassays with young bolls of ten $F_1$ cotton hybrids**

| $F_1$ Hybrids | Mortality % days after sowing at | | | |
|---|---|---|---|---|
| | 60 | 90 | 120 | Mean |
| CRIS-134 × MG-6 | 80 | 60 | 33 | 57.7 |
| BH-167 × MG-6 | 80 | 60 | 33 | 57.7 |
| CRIS-134 × IR-NIBGE-1524 | 80 | 60 | 27 | 55.7 |
| FH-941 × IR-NIBGE-1524 | 80 | 40 | 33 | 51.0 |
| CRIS-134 × IR-NIBGE-3701 | 77 | 40 | 33 | 50.0 |
| CIM-573 × IR-NIBGE-1524 | 77 | 60 | 33 | 56.7 |
| CIM-573 × IR-NIBGE-3701 | 75 | 58 | 32 | 55.0 |
| CRIS-134 × FH-113 | 73 | 55 | 31 | 53.0 |
| BH-167 × AA-802 | 73 | 54 | 29 | 52.0 |
| CRIS-342 × IR-NIBGE-3701 | 70 | 52 | 28 | 50.0 |

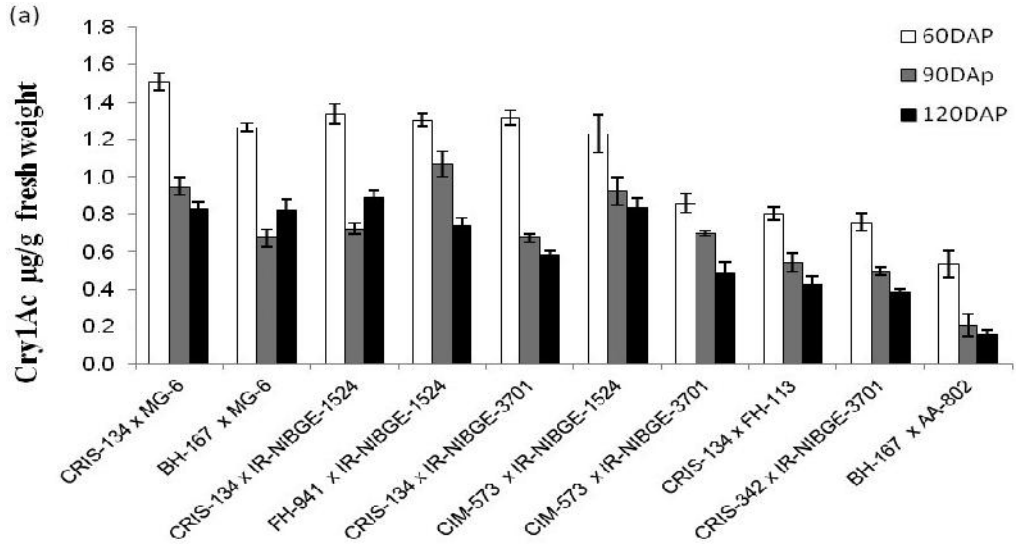


**Fig 1. Expression of gene Cry1Ac in top ten crosses at different crop growth periods**

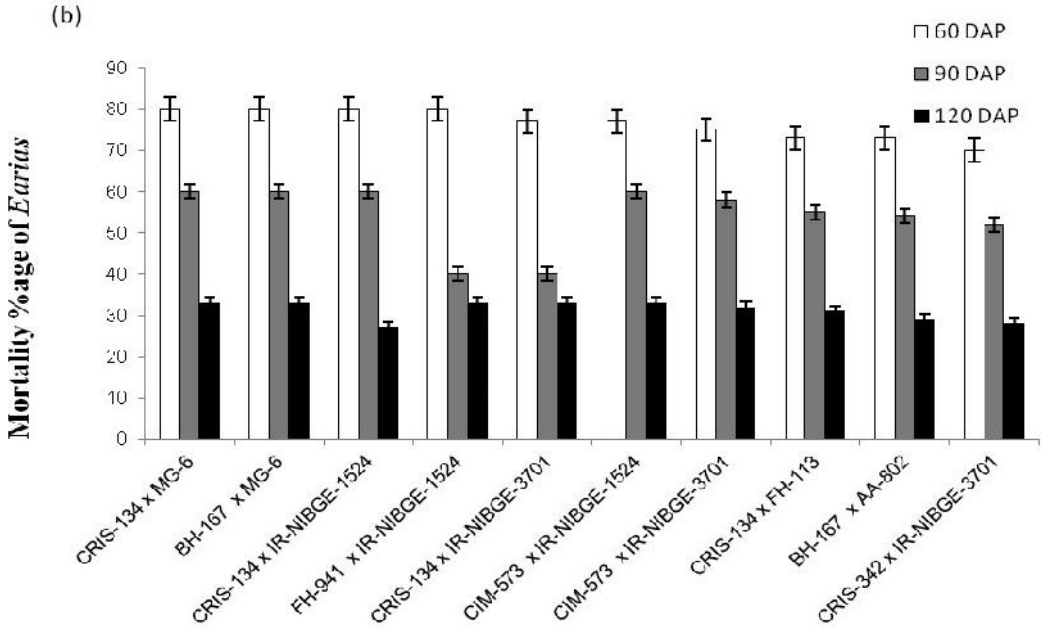


**Fig 2. Mortality percentage of *Earias* larvae at different crop growth stages in ten $F_1$ hybrids**

## DISCUSSION

Optimum gene expression level of Cry1Ac in Bt cotton is required for better control of bollworms. The present studies confirmed the significance of cross combinations for developing Bt Cotton varieties for optimum gene (Cry1Ac) expression level required for better bollworm control despite having a common gene insertion event. The Cry1Ac content was high at 60 DAP. Later on the expression declined throughout the season and reached minimum at 120 DAP. However, the Cry1Ac protein did not reach to the undetectable level. Decrease in gene expression over the crop period has been well documented (Greenplate, 1999; Olsen *et al*., 2005; Poongothai *et al*., 2010; Wan, *et al*., 2005) and present findings are in corroboration with the early reports. Studies undertaken by various scientists also revealed that there was considerable intra-plant variability in expression of cry toxin. According to Greenplate (1999) terminal leaves expressed very high level of concentration compared to proximal fruiting structure. Udikeri (2006) reported that the Cry protein expression was maximum in leaves followed by squares, flowers and bolls. Greenplate (1999) reported the decline in Cry1Ac concentration in terminal of the plant from 63.4-34.5 µg/g at 53 and 116 days after sowing. Similarly seasonal variation in Cry1Ac was observed by Adamczyk *et al*.

(2004) where reduce in concentration from more than two to one ppm was quiet evident. Further studies of Sun *et al*. (2002) and Wan *et al.* (2005) also confirmed the fact that expression pattern vary within the season with maximum concentration at the start and least at the end. Water stress has also been reported to affect expression of transgenes in transgenic crops such as maize (Trarore *et al.,* 2000), peas (Sousa *et al.,* 2004) and cotton (Benedict *et al.,* 1996; Luo *et al.,* 2008; Rochester 2006). This has serious implications: (i) ineffective pest control; (ii) pest becoming resistant to the Bt toxin, and (iii) high pesticide use. Kranthi *et al*., (2005) demonstrated that the toxin expression declined with crop age in all the Bt hybrids tested. Furthermore, as Bt cotton plants age, toxin concentrations decline, which could increase survival of pests that have inherently low susceptibility to Bt toxins (Carrière *et al*., 2010; Brévault *et al*., 2012).

Mortality of bollworms also depend with the blend insecticidal endotoxin by the expression of Cry genes, it was recorded high during early season and gradually decline with passage of time. Low toxin level may not only cause poor pest control and but also increase the chances of resistance development in cotton insects against Cry protein (Tabashnik *et al*., 2008). The present findings are in close agreement with the result of earlier studies that the utility of GM cotton is related to the expression level of endotoxin (Gutierrez *et al*., 2006) that remains inconstant throughout the plant life cycle (Olsen *et al*., 2005). Lower gene expression will not only reduce the cotton yield but also enhance the risk of cross resistance development in bollworms. To reduce the chance of cross resistance development in bollworms, varieties having low level of Cry1Ac toxin should not be recommended. Pakistani cotton genotypes anchorage only Cry1Ac gene. Keeping in view the current gene expression in our commercial varieties, Agriculture Extension department in Pakistan recommend farmer's for regular pest scouting at weekly intervals during the critical fruiting period of the crop, with more emphasis on locating larvae in fruiting parts for better management of the cotton crop.

The current finding confirmed that the selection of parental background is important for sustainable level of Cry1Ac gene expression. Therefore, seed provider companies should evaluate their hybrids judiciously for highest levels of expression in fruiting parts and also for relatively effective level of toxin expression late in the crop period. Since the commercialization of Bt transgenic technology it has been proven that it's the most environment friendly methods of bollworm control, it is important for the technology itself that researchers and technology supplier must provide best quality products to farmers which gives the better bollworms control and ultimately better yield.

The current finding confirmed that the selection of parents is important for tenable level of Cry1Ac gene expression. Consequently, seed provider companies should assess their hybrids judiciously for maximum expression of toxic levels in fruiting parts and as well for comparatively effectual level of toxin expression delayed crop period. Since the commercialization of Bt transgenic technology it has been proven that it's the most environment friendly method of bollworm control, it is important for the technology itself that researchers and technology supplier must provide best quality products to farmers which gives the better bollworms control and ultimately better yield.

**Acknowledgements:** I deem it my utmost pleasure in expressing profound benedictions to Dr. Muhammad Naveed, Senior Scientific Officer and head of Entomology Section, Central Cotton Research Institute, Multan for providing an additional support at every step of research. I extend my deep emotion of appreciations, gratitude and indebtedness for his valuable guidance

## REFERENCES

Acharjee, S., and B. K. Sarmah (2013). "Biotechnologically generating 'super chickpea' for food and nutritional security". Plant Sci. 207: 108–116.

Adamczk, J. J. and D.V. Sumerfold (2001). Potential factors impacting season long expression of Cry1Ac in 13 commercial varieties of Bollgard Cotton. J. Insect Sci. 1-13.

Adamczyk, J.J., D. D.Hardee, L.C. Adams, and D.V. Sumerford (2001). Correlating differences in larval survival and development of bollworm (Lepidotera: Noctuidae) and fall armyworm (Lepidotera: Noctuidae) to differential expression of Cry1Ac delta endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol. 94: 284-290.

Adamczyk, J.J., R. Willium, and J. Meredith (2004). Genetic bases for viability of cry1Ac expression among commercial transgenic *Bacillus thuringensis* (Bt) cotton cultivars in the Unites States. J. Insect Sci. 8: 17-23.

Adamczyk, J.J., J.O. Perera, and M.R. Meredith (2009). Production of mRNA from the Cry1Ac transgene differs among bollgard lines which correlate to the level of subsequent protein. Transgenic Res., 18:143-149.

Bakhsh A., A. Q. Rao, A.A. Shahid, T. Husnain, and S. Riazuddin (2010). CaMV35S is a developmental promoter being temporal and spatial in expression pattern of insecticidal genes (Cry1Ac & Cry2A) in cotton. Aust. J. Basic Applied Sci., 4:37-44.

Benedict, J. H., E. S. Sachs, D. W. Altman, W. R. Deaton, R. J. Kohel, and S. A. Berberich, (1996). Field performance of cottons expressing Cry1A insecticidal proteins for resistance to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. 89: 230–238.

Brévault T, J. Achaleke, S. Nibouche,and Y. Carrière *(*2012*).* Assessing the role of non-cotton refuges in delaying *Helicoverpa armigera* resistance to Bt cotton in West Africa*. Evol. Appl. 5(1):*53–65.

Carrière Y, D.W. Crowder, and B.E. Tabashnik *(*2010*).* Evolutionary ecology of insect adaptation to Bt crops*.* Evol. App. l3 (*5-6*):*561–573*.

Crickmore N., D.R. Zeigler, E. Schnepf, J. Van Rie, D. Lereclus, L. J. Baum, A. Bravo, and D.H., Dean (2005). *Bacillus thuringiensis* Toxin Nomenclature (Homepage). [cited January 2010].

Chen S, J.Y. Wu, X.L. He, J.Q. Huang, B.L. Zhou, and R.X. Zhang (1997). Quantification using ELISA of *Bacillus thuringiensis* insecticidal protein expressed in the tissue of transgenic insect resistant cotton. J. Jiangsu Agric. Sci. 3: 154–156.

Greenplate, J.T. (1999). Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. J. Econ. Ent. 92 : 1377-1383.

Greenplate, J.T, S.R. Penn, J.W. Mullins, and M. Oppenhuize (2000). Seasonal Cry1Ac levels in DP50B: the Bollgard basis for Bollgard II. In: Dugger P, Richter R (eds) Proceedings, beltwide cotton conference, San Antonio, TX, 4–8 Jan 2000. National Cotton Council of America, Memphis, TN: 1039–1041.

Gutierrez, A.P., J.J. Adamczyk, S. Ponsard, and C.K. Ellis (2006). Physiologically based demographics of *Bt* cotton-pest interactions II. Temporal refuges, natural enemy interactions. Ecol. Model. 191: 360–382

Holt, H. E., C. Mares and R. Akhurst (2002). Determination of the Cry Protein Content of Bt Transgenic Cotton: a Technical Manual for Laboratory Use. Canberra: CSIRO Australia, Division of Entomology.

Kranthi, K.R., S. Naidu, C.S. Dharwad, A.Tatwawadi, K. Mate, E. Patil, A.A. Bharose, G.T. Behera, R.M. Wasaskar, and S. Kranthi ( 2005). Temporal and intra-plant variability of cry1Ac expressing in Bt-cotton and its influence on the survival of cotton bollworm, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera). Curr. Sci. 89: 291- 298.

Liu, Y.B., J.S. Li, C.Y. Zhao, N.W. Xiao, and X. Guan (2012). "Occurrence and ecological consequences of transgenic rice gene flow: a review". 23 (6): 1713–20.

Llewellyn, D., Y. Cousins, A. Mathews, L. Hartweck, and B. Lyon (1994). Expression of *Bacillus thuringiensis* insecticidal protein genes in transgenic crop plants. Agric. Ecosyst. Environ. 49: 85-93.

Luo, Z., H. Dong, W. Li, Z. Ming and Y. Zhu. (2008). Individual and combined effects of salinity and waterlogging on Cry1Ac expression and insecticidal efficacy of Bt cotton. Crop Prot. 27: 1485–1490.

McLean, M (2011). "A review of the environmental safety of the Cry1Ab protein". Environ Biosafety Res. 10 (3): 51–71.

Naveed, M., M. Rafiq, T. Jan, R.Saeed., S.Wazeer., K.Hussain, and A.Rana (2014). "Earias spp survival to transgenic Bt-cotton strains having different protein levels" Report submitted to Bio Safety Research in Pakistan Grant Program.

Olsen, K.R., J.C. Daly, H.E. Holt, and E.J. Finnegan (2005). Seasonal-long variation in expression of Cry1Ac gene and efficacy of *Bacillus thuringensis* toxin in transgenic cotton against *Helicoverpa armigera* Hub. (Lepidoptera : Noctuidae). J. Econ. Ent. 98: 1007-1017.

OECD. (2007). Consensus Document on Safety Information on Transgenic Plants Expressing *Bacillus thuringiensis* - Derived Insect Control Protein. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 42. Organisation for Economic Co-operation and Development, Paris.

Qaim, M. (2009). "The economics of genetically modified crops", Annual Review of Resource Economics. 1: 665–69.

Poongothai S., R. Ilavarasan, and C.M. Karrunakaran (2010). Cry1Ac levels and biochemical variations in Bt cotton as influenced by tissue maturity and senescence. J. Plant. Breed., Crop Sci. 2: 96-103.

Rochester, I.J. (2006). Effect of Genotype, Edaphic, Environmental Conditions, and Agronomic Practices on Cry1Ac Protein Expression in Transgenic Cotton. J.C.Sci. 10: 252-262

Sousa-Majer, M. J., N. C, Turner, D. C, Hardie, R. L, Morton, B. Lamont, and T. J. V. Higgins (2004). Response to water deficit and high temperature of transgenic peas (Pisum sativumL.) containing a seed-specific amylase inhibitor and the subsequent effects on the pea weevil (Bruchus pisorumL.) survival. J. Exp. Bot. 55: 497–505.

Sun, C.G., J. Xu, Q.W. Zhang, H.B. Feng, F. Wang, and R. Song (2002). Effect of transgenic Bt cotton

on population of cotton pests and their natural enemies in Xinjiang. Chinese J. Biol. Control, 18: 106-110.

Tabashnik, B.E., A.J. Gassmann, W.D. Crowde,r and Y. Carriere (2008). Insect resistance to *Bt* crops: evidence versus theory. Nat. Biotechnol. 26: 199–202

Trarore, S. B., R. E, Carlson, C. D. Pilcher, and M. E, Rice (2000). Bt and non-Bt maize growth and development as affected by temperature and drought stress. Agron. J. 92: 1027–1035.

Udikeri, S. S. (2006). Evaluation of new generation Bt genotypes, sustainability of Cry protein expression, computation of ETL, effect on aphid predators and development of IPM model for Bt cotton under rainfed condition. Ph. D. Thesis (unpublished). Univ. Agric. Sci., Dharwad (India).

Wan, P., Zhang, Y., Kongming, and M. Huang (2005). Seasonal expression profiles of insecticidal protein and control efficacy against *Helicoverpa armigera* for Bt cotton in China. J. Econ. Ent., 98: 195-201.