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EFFECT OF NATURAL HIGH TEMPERATURE AND FLOODING CONDITIONS ON *Cry1Ac* GENE EXPRESSION IN DIFFERENT TRANSGENIC Bt COTTON (*GOSSYPIUM HIRSUTUM* L.) CULTIVARS

SAMINA JAM NAZEER AHMAD[1,2], DILAWAR MAJEED[1], ABID ALI[1], MUHAMMAD SUFIAN[1], ZUBAIR ASLAM[3], MUJAHID MANZOOR[1] AND JAM NAZEER AHMAD[1,2*]

[1]*Dr. Jam Laboratory, Department of Entomology, University of Agriculture Faisalabad, Pakistan*
[2]*Plant Stress Physiology and Molecular Biology Lab, Department of Botany, University of Agriculture Faisalabad, Pakistan*
[3]*Department of Agronomy, University of Agriculture Faisalabad, Pakistan*
[*]*Corresponding author's email: jam.ahmad@uaf.edu.pk*

Abstract

A severe abiotic and biotic stress was faced to cotton growers in South Punjab due to high temperature and high rain fall occurred during 2014. In current study, variation in the *Cry1Ac* gene expression from samples collected from five Bt (IUB-212, CIM 616, CIM-598, Lalazar, FH-183 containing *Cry1Ac* insecticidal gene) and one non Bt cotton (MNH 786) cultivars cultivated in three districts (Multan, Bahawalpur and Rahim Yar khan) of Southern Punjab were investigated. The investigated varieties were also cultivated under optimal greenhouses controlled conditions for expression analysis of *Cry1Ac*. The samples were taken to IGCDB laboratory for *Cry1Ac* gene expression analysis through quantitative RT-PCR and ELISA. The results indicated the abrupt variation in the expression of cry 1Ac gene in samples collected from environmental stressed districts of Punjab. Combined stress of high temperature and humidity variation resulted the down-regulation of Bt protein expression as compared to control greenhouse grown plants. The Bt cotton control efficacy was considerably reduced due to combined stress. It was concluded that reduced expression of *Cry1Ac* during 2014 was due to adverse environmental condition attributed to the degenerated levels of Bt endotoxin protein. This is the first report of the influence of abiotic stresses on the expressional variation in cry 1Ac and ultimately high infestation of insect pest on cotton in Pakistan.

Key words: *Cry1Ac* gene, Flooding, High temperature, Bt cotton, Stress conditions.

Introduction

Cotton is industrial crop and most importantly fiber crop known as 'White gold'. It exists at 2nd number after soybean as an oilseed crop (Ali *et al*., 2010). Currently the Bt cotton expressing *Bacillus thuringiensis* (Bt) derived toxin from cultivated on a huge area as a marketable scale in several cotton producing nations counting China (Dong *et al*., 2004), Australia (Whitehouse *et al*., 2005), USA (Adamczyk & Meredith, 2004), Mexico, Colombia, India, South Africa, Argentina and Pakistan (James, 2005; Cheema *et al*., 2016). Transgenic cotton producing Cry genes such as only *Cry1Ac* or in combination *Cry1Ac+ Cry2Ab* are known as effective cotton varieties in controlling significant lepidopteran pests. Such type of varieties are favorable to environment and farmer by decreasing application of pesticides and conserving strains of valuable insects (Pray *et al*., 2002; Tabashnik *et al*., 2002). In the field conditions, widespread growing of transgenic varieties has not indicated collapse in monitoring lepidopteran insect pests (Dong & Li, 2007; Tabashnik *et al*., 2008). While numerous publications specify the Cry protein effectiveness against insect of Lepidoptera is not remain reliable throughout season (Olsen *et al*., 2005). But in recent past *P. gossypiella* has evolved resistance to transgenic cotton producing *Cry1Ac* (Ojha *et al*., 2014; Tabashnik *et al*., 2014; Wang *et al*., 2016) as well as pest management practices were suggested to combact resistances issues (Ahmad *et al.,* 2020ab).

A number of plants have advanced to flourish in different environmental conditions, tolerating various stresses (biotic and abiotic) in amalgamation. Different plants have evolved various significant processes that permit them to perceive accurate environmental deviations and respond them, decreasing damage and saving the resources for enlargement and reproduction of various plantations (Rizhsky *et al*., 2004; Malik *et al.,* 2009). The previous studies revealed that outcomes of exploring stress factors in separately do not elucidate more than one stress in plants (Ahmad *et al.,* 2013; Ahuja *et al.,* 2016). In the current circumstances modern techniques for noticing stress tolerant plants under individual or combined stress may be insufficient (Mittler & Blumwald, 2010). The reported work indicated the signaling pathways interacting antagonistically (Anderson *et al*., 2004; Asselberg *et al*., 2008) under biotic and abiotic environmental conditions (Ahmad *et al.,* 2014; Ahuja *et al.,* 2016; Ihsan *et al.,* 2017).

Previous investigations advised that pest management efficiency of transgenic cotton varies among different cultivars considering plant ages (Adamczyk & Sumerford, 2001) and parts (Abel & Adamczyk, 2004; Wan *et al*., 2005) under stresses (Rao, 2005). Though, recognition of transgenic plant responses to environmental stresses predominantly exerts different effects on Cry protein production level and the subsequent deviation of lepidopteran pests control efficacy is found very limited.

Exposure of transgenic cotton in field conditions experiences massive abiotic and biotic stresses that harmfully affect development, growth pattern and yield. Mainly abiotic stresses are involved in Cry gene expression levels in Bt cotton plants and target efficacy of cotton against insect pests (Mahon *et al*., 2002). As an intanse, in case of increased doses of nitrogen fertilizers increased the *Cry1Ac* toxin expression as compared to decreased doses or nitrogen deficiency, elevated CO_2 exerts adverse effects in significant decrease of *Cry1Ac*

toxin levels (Coviella *et al*., 2002; Chen *et al*., 2005; Pettigrew & Adamczyk, 2006). Treatment of Nacl significantly reduced Bt toxin production (Jiang *et al*., 2006). Growth of Bt plants under low and high temperature or water scarcity considerably reduced *Cry1Ac* production levels (Benedict *et al*., 1996; Chen *et al*., 2005; Dong & Li, 2007; Rana *et al*., 2015) raised temperature and salinity also decreases *Cry1Ac* expression (Luo *et al*., 2008: Aamir *et al*., 2015). In recent studies it was also noted that high temperature reduces the concentration of Bt toxin during development of boll formation (Yuan *et al*., 2012; Zhang *et al*., 2012).

Transgenic cotton efficiency is dependent upon cry genes expression through insecticidal protein synthesis in Bt cotton, if the endotoxin level decreased then the evolution of insect resistance to transgenic increased effecting the proper management of targeted pest of cotton (Rao, 2005; Kranthi *et al*., 2005; Gutierrez *et al*., 2006; Tabashnik *et al*., 2008). Consequently abiotic stress conditions disturbing endotoxin production mechanisms essential to understand the resistance management approaches to delay resistance evolution.

Abiotic stresses have seasonal impacts on all plants mainly cotton plants are also included (Kahlown and Azam, 2002; Barrett-Lennard, 2003; Zhou *et al*., 2015). Efficacy of transgenic cotton may be linked with secondary compounds particularly under different environmental stress conditions. Researchers noted that gossypol presence in transgenic cotton expressively increased the Cry1Ab toxin efficacy against *Helicoverpa armigera* and *Heliothis virescens* (Zhang *et al*., 2002). In contrast increased level of tannins expressively reduces efficiency of Cry genes (Daly & Fitt, 1998). Consequently significant alterations of Cry proteins may elaborate variations in the efficacy of transgenic cotton under stresses. Recently, molecular research was conducted on disease transmitting (Sharif *et al.,* 2019; Malik *et al.,* 2020) and yields reducing pest of different crops (Ahmad *et al.,* 2019). In the present study, one non-Bt and five transgenic Bt cotton cultivars were naturally subjected to combined stresses of high temperature and flooding at the initial seedling stage during 2014. The *Cry1Ac* gene expression was measured by implementing the RT-PCR and Bt protein content with the help of quantitative ELISA.

Material and Methods

Plant source: Acid-delinted Seeds of Bt cotton cultivars particularly (IUB-212, CIM 616, CIM-598, Lalazar, FH-183 and non Bt MNH 786 were sown in three districts of Southern Punjab, Pakistan including Multan, Bahawalpur and Rahim Yar Khan during 2014. During 2014 naturally the high rain fall and temperature ensureed increased humidity and produces waterlogging like conditions and raised temperature. So in this way the cotton plants were naturally subjected to combined stresses of both high temperature and waterlogging conditions. These varieties were grown in a greenhouse as a control under optimum environmental conditions from April to August at PARS, University of Agriculture Faisalabad. The above mentioned varieties grown in different districts and under greenhouses used for taking fresh samples of leaf, squares and bolls for expression analysis of *Cry1Ac* and for insect bioassays. The samples from Bt and non Bt cultivars were taken at the age of 60 days. Three plants were selected randomly from each genotype to take the sample of leaf, squares and bolls for bioassay.

***Cry1Ac* protein quantification:** To measure the level of Cry1Ac present in the leaves, squares and bolls sample, Cry toxin quantification Kit (QuantiPlate™ Kit, EnvroLogix, Inc., Portland, ME) was used in Enzyme-Linked Immuno-Sorbent Assay (ELISA). In ELISA, the intensity of production of color is directly proportional to the *Cry1Ac* level in the extract of samples. A standard curve with calibrators supplied with Kit indicated optimal densities for all samples. The quantity of *Cry1Ac* was calculated in ppm corresponding to (µg/g) of fresh weight of the leaf.

Expression of *Cry1Ac* by polymerase chain reaction (PCR)

RNA extraction: Total RNA from 0.2g to 0.5g of leaves, squares and bolls of Bt and non Bt plants were extracted in liquid nitrogen by the TriZol LS-Reagent® method (Invitrogen- Carlsbad, MI, USA). Briefly, the ground powder was mixed well with 1 ml of TriZol LS-Reagent® and nucleic acids were dissolved in 15 µl sterile deionized water. After extraction, for purification, RNAs 8µg-12µg from samples in a total volume of 50 µl using 1X reaction buffer were treated with RNase- Free DNase (5 units) of RQ1 (Promega, Madison, WI, USA) and placed at 37°C for 1 h (Promega, Madison, WI, USA). The purified RNA was eluted in ddH20 and concentration was measured by pico200 at λ =260 nm.

Reverse transcription: After purification**,** 1µg of pure RNA was used for the synthesis of complementary DNA (cDNA) by using 5µM oligodT18 primer, Reverse Transcriptase (200 units) of Superscript® II (Invitrogen, Carlsbad, CA, USA) in a 25 µl reaction mixture (10 mM DTT, 20 µM dNTPs and 40 units of RNase Out™ (Invitrogen) following protocol (Ahmad *et al*., 2014). The mixture (RNA+oligodT18 or specific primer) were heat-denatured at 65°C for 5 min before adding the reverse transcriptase and the other components. For controls, no reverse transcriptase, were used to test the efficiency of the DNase treatment.

RT-PCR amplification: Amplification of *Cry1Ac* and other defensive genes into the genome of transgenic cotton plants were performed using polymerase chain reaction. Complementary DNA (cDNA) of transgenic cotton plants were used for amplification of Bt genes, cDNA of non-transgenic plant was used as a negative control. Real time PCR amplification was done from diluted RT reaction mixture (1/10) in the presence of *Cry1Ac* primers (2µM) each forward and reverse primers and Taq DNA polymerase (1.5 units) (Promega). The PCR cycles conditioned following 45 seconds denaturation step at 94°C, 45 seconds annealing step at 56°C and 45 seconds elongation steps at 72°C for 34 cycles. The final extension of 10 minutes at 72ºC was used for final extension. Amplified PCR products (8 µl) of *Cry1Ac* genes were run on 2% agarose gel using 1kb DNA ladder

Marker. The gel was visualized under UV light using SYNGENE gel documentation system.

Statistical analysis

The student t-test was used to study gene expression of Bt and control non Bt cultivars.

Results

Metrological condition during 2014 and *Cry1Ac* gene expression: The transgenic Bt cotton requires normal environmental conditions to express the Cry genes. The recorded temperature for normal growth, development and gene expression was 32ºC and 50-80% relative humidity. But in 2014, the average increased temperature level was recorded high in different observed districts of Punjab (RY Khan, Bahawalpur and Multan) (32-40 C) exerting adverse effects on *Cry1Ac* gene expression. The variation in humidity also disturbed the normal *Cry1Ac* gene expression. In 2014, due to high rain fall and high temperature (>40C) during cotton early development stages leaded to severe stress condition in South Punjab. In short, the combined effect of all above mentioned abiotic environmental factors suppressed the *Cry1Ac* gene expression and ultimately high attack of *P. gossypiella* and white fly was observed. (Table 1) showed average annual increase in temperature and humidity throughout country in 2014.

Cry1Ac gene expression in leaves, squares and bolls of cotton plant under laboratory normal environmental conditions: We observed the *Cry1Ac* production levels in different plant parts under normal abiotic environmental conditions. Maximum *Cry1Ac* gene expression was recorded in leaves as compared to squares and bolls in different cotton cultivars except Non-Bt MNH-786 (0±0.0), IUB-212 (0.70±0.23), CIM-616 (1.04±0.17), CIM-598 (1.05±0.29), Lalazar (1.00±0.11) and FH-183 (0.65±0.23). The significant gene expression was recorded in Bt cotton squares counting Non-Bt MNH-786 (0±0.0), IUB-212 (0.47±0.18), CIM-616 (0.61±0.15), CIM-598 (0.49±0.21), Lalazar (0.48±0.23) and FH-183 (0.39±0.11) respectively. Whereas the lowest non-significant Cry1Ac gene expression was recorded in cotton bolls of Bt cultivars including Non-Bt MNH-786 (0±0.0), IUB-212 (0.24±0.07), CIM-616 (0.54±0.24), CIM-598 (0.32±0.06), Lalazar (0.40±0.13) and FH-183 (0.31±0.05)**.** The highly significant expression recorded in CIM-598 was (1.05±0.29) in leaves which was higher as compared to square (0.49±0.21) and bolls (0.32±0.06). While the least *Cry1Ac* expression recorded in FH-183 which was high in leaves (0.65±0.23) as compared to square (0.39±0.11) and bolls (0.31±0.05). The results indicate that endotoxin protein concentration was found high in leaves a compared to other plant parts. The control non-Bt plants were not able to express Bt proteins (Fig. 1).

Cry1Ac gene expression under stress field environmental condition: Previously a number of researchers worked on plant stress in different crops and concluded that severe stresses exerts adverse effects on plant growth, development and gene expression (Wang *et al*., 2003; Rana *et al*., 2015). Under severe stress including high temperature and flooding conditions exerts adverse effects on *Cry1Ac* gene expression in different plant parts. Here we noted the decreased gene expression in leaves of Bt cultivars from samples collected from RY Khan (IUB-212 (1.40±0.23), CIM-616 (1.84±0.28), CIM-598 (2.00±0.22), Lalazar (1.75±0.35) and FH-183 (1.33±0.27) with no expression in Non-Bt MNH-786 (0±0.0). In the same way in plants collected from Bahawalpur was as Non-Bt MNH-786 (0±0.0), IUB-212 (2.52±0.31), CIM-616 (2.76±0.39), CIM-598 (2.98±0.75), Lalazar (2.64±0.21) and FH-183 (2.27±0.23). Further, the decreased Bt gene expression was also noted in all Bt cultivars from samples collected from Multan fields as Non-Bt MNH-786 (0±0.0), IUB-212 (1.47±0.29), CIM-616 (1.87±0.20), CIM-598 (1.94±0.52), Lalazar (1.94±0.24) and FH-183 (1.85±0.46).Under severe conditions the *Cry1Ac* gene expression was decreased significantly in leaves collected from all three districts (Fig. 2).

Table 1. Metrological data observed during year 2014 throughout country.

Region	Coordinates	Coordinates	R.H.	Annual temp.
Bahawalpur	29°24'N	71°40'E	37%	32-40
Multan	31°40'N	71°05'E	36%	35-40
Dera Ghazi Khan	30°05'N	70°43'E	38%	28-36
Muzaffargarh	30°05'N	71°14'E	40%	30-38
Rahimyar Khan	28°30'N	70°25'E	37%	31-40
Layyah	23°54'S	21°55'E	39%	33-40
KotAddu	30°30'N	71°00'E	40%	32-38
Liaqatpur	29°30'N	70°30'E	37%	31-39
Dera Ismail Khan	31°50'N	70°50'E	35%	25-32
MirpurKhas	25°30'N	69°0'E	49%	31-41
Nawabshah	26°15'N	68°25'E	41%	32-41
Pano Akil	27°51'N	69°07'E	25%	39-40
Rohri	27°45'N	68°51'E	37%	31-41
Sukhar	27°0'N	42°0‘E	38%	33-41
Khairpur Nathan Shah	27°06'N	67°44'E	36%	36-42
Ghotki	28°05'N	69°21'E	30%	39-41
(Khuzdar)	27.81°0'N	66.61°0'N	49%	24-32
Panjgoor	27°0'N	64°05'E	23%	27-35

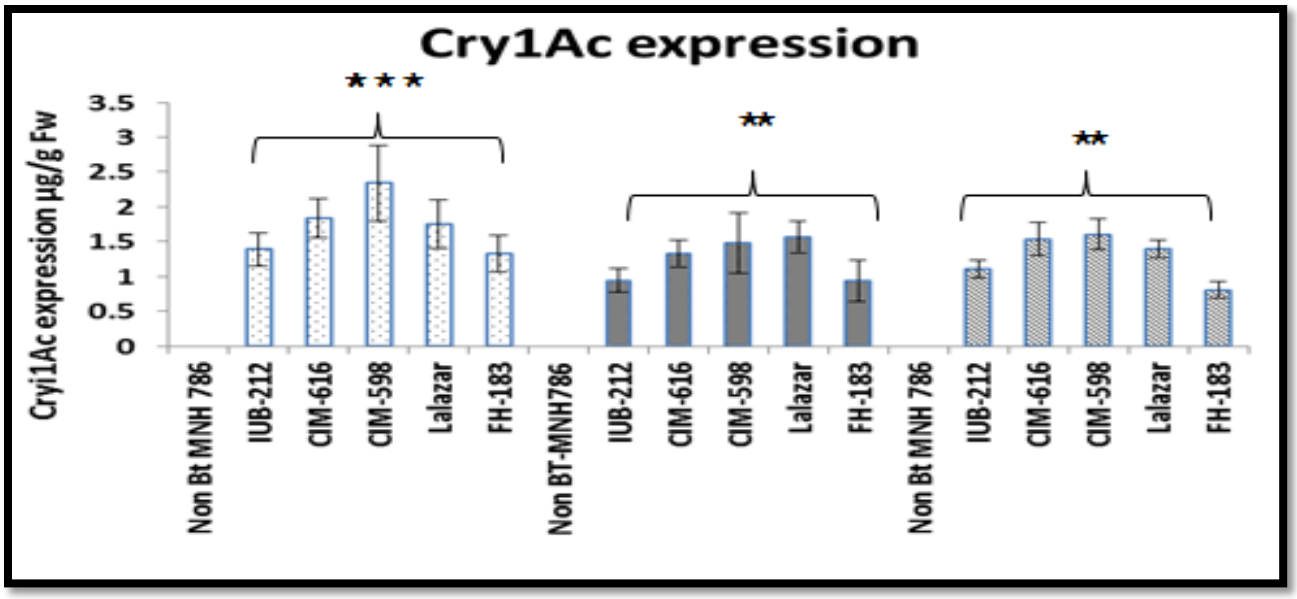

Fig. 1. Cry1Ac gene expression in cotton leaves (White bars), square (dark grey) and bolls (grey bars) of samples collected from cotton plants cultivated under laboratory condition ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.005$).

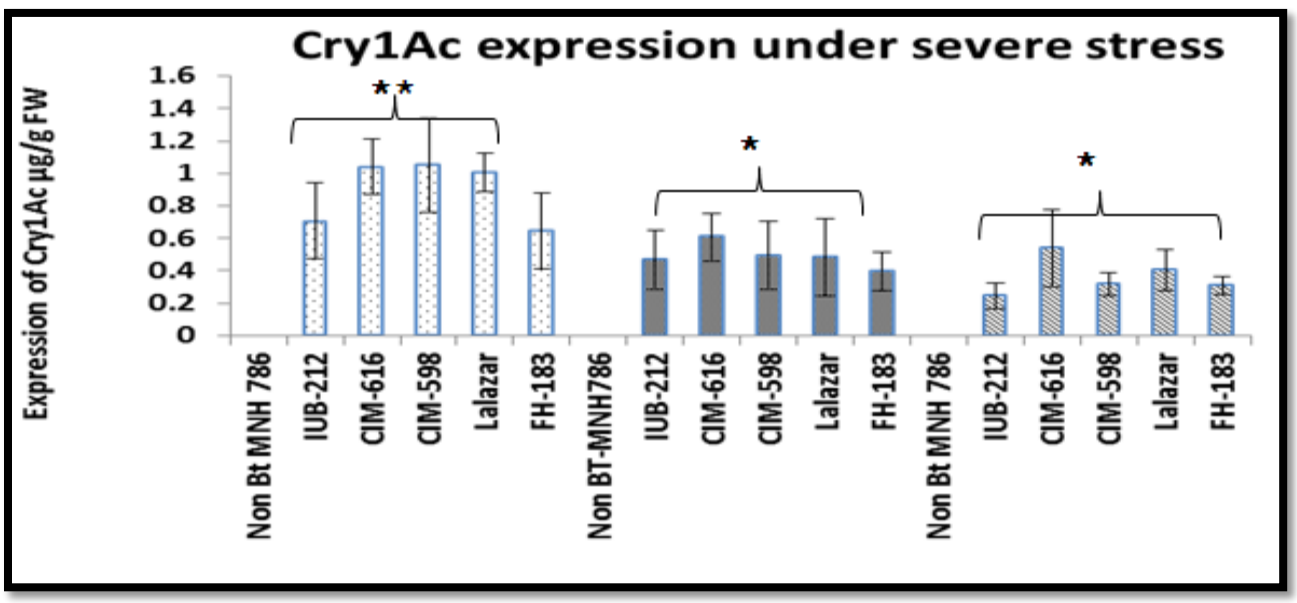

Fig. 2. Cry1Ac gene expression in leaves of cotton plants collected from cotton fields of RY Khan (White bar), Bahawalpur (dark grey bar) and Multan (Grey bars) faced stress field environmental conditions (2014) ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.005$).

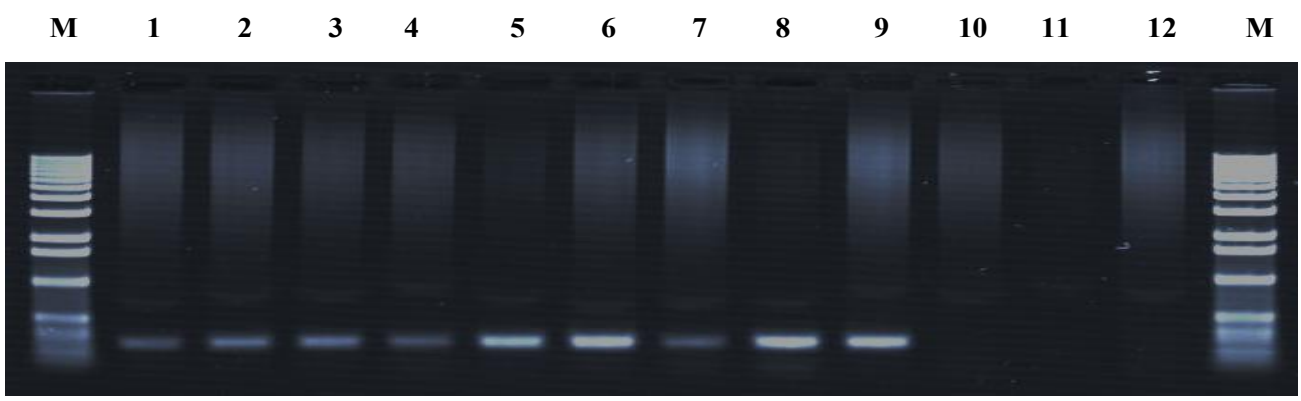

Fig. 3. Gene expression through RT-PCR by using Cry1Ac specific primers (162bp) in flower buds and bolls of Bt cultivars. Lane1-2 Bt FH-142 flower bud and boll; Lanes 3-4- Bt IUB-212 flower and boll; Lanes 5-6 CIM 598 Flower bud and bolls, Lanes 7-8 Bt Lalazar; bud and boll; Lane 9- Positive control; Lane 10- Non-Bt 786 flower bud; Lane 11- Non Bt 786 boll; Lane12- H20; Lanes M- 1kb molecular ladder (Invitrogen).

Expression of Cry1Ac genes through RT-PCR: Gene expression through RT-PCR was confirmed using *Cry1Ac* specific primers in flower, buds and bolls of Bt cotton cultivars cultivated under laboratory conditions. The significant expression shown by CIM-598 in flowers buds and bolls was observed in Lane 5-6. In the same way, lalazar cultivar also depicted intermediate gene expression in lane 7-8. FH-183 and IUB-212 were recorded as least *Cry1Ac* gene expressive cultivar as indicated in lane 1-2 (Fig. 3). The results of Cry1Ac expression are in accordance with RT-PCR data.

Discussion

Abiotic environmental factors such as salinity, cold, heat, nutrient, waterlogging and drought stresses exerts massive effect on global agriculture and reduce average yields more than 50% for major economic crops (Coviella *et al*., 2002; Wang *et al*., 2003). Harsh environmental stresses affect severely the growth and yield of different crops in natural conditions (Boyer, 1982). The expression of transgenes is considerably affected due to environmental stress in genetically modified crops. For example Bt maize affected by low levels of nitrogen and water exerted stresses (Traore *et al*., 2000; Bruns & Abel, 2003). High temperature affects dihydroflavonol reeducates in transgenic petunia and genes encoding for polygalacturonase and pectin methylesterase in transgenic tomato (Meyer *et al*., 1992; Lurie *et al*., 1996). Water deficit stress exerts adverse effects on r-amylase inhibitor in transgenic peas (Sousa-Majer *et al*., 2004). The current work was evaluated that mutual effect of both high temperature and waterlogging conditions, significantly lowered endotoxin protein and Bt cotton effectiveness against bollworms. The high temperature and waterlogging conditions reduced Bt protein content in all Bt cultivars, which results in non-significant *P. gossypiella* population reduction control efficacy.

It is demonstrated that cotton plant growth was found highly temperature dependent (Downton & Slatyer, 1972). It requires diverse temperature range at different growth stages such as at flowering and boll stage high temperature is compulsory (Reddy *et al*., 1991, 1992a) while the low temperature exerts severe effects on fruiting branches (Hodges *et al*., 1993; Reddy *et al*., 1992b; Roussopoulos *et al*., 1998). In contrast increased high temperature affects growth and yield of crop by disturbing osmotic pressure and plant metabolism (Rana *et al*., 2015). While the waterlogging exerts considerable damage to rooting volume, inhibition of respiration and nitrogen uptake which lead to biochemical and physiological disturbance was noted due to lack of oxygen (Jackson & Drew, 1984; Meyer *et al*., 1987; Dong *et al*., 2002; Sairam *et al*., 2008). Combination of both high temperature and waterlogging conditions exerts more severe stress and causes more harm to plants compared to other abiotic factors (Barrett Lennard, 2003).

Though these both stresses entangle diverse mechanisms for harming the Bt cotton, involved changed nitrogen metabolisms, decomposition and synthesis of protein have been recorded (Hocking *et al*., 1985; Miller & Dennis, 1996; Hodges *et al*., 1993; Roussopoulos *et al*., 1998). In the current well designed experiment, high temperature and waterlogging conditions lead to considerable reduction in protein content. It may take place due to restricted uptake of nitrogen and synthesis of protein or interference with proline base proteins (Ihsan *et al.,* 217) or increased decomposition of protein under severe stresses (Gouia *et al*., 1994; Pessarakli & Tucker, 1985; Hocking *et al*., 1985).

The current study is done to verify all transgenic cotton cultivars for Bt *Cry1Ac* gene through immunostrip method except one control non Bt cultivar. For proper protection of crop from various insect pests requires a precise level of endotoxin protein at specific stage and time such as 1.8µg/g fresh weight was recommended for lepidopteran pests (Kranthi *et al*., 2005). Five Bt cotton selected genotypes IUB-212, CIM-598, CIM-616, Lalazar and FH-183 depicting significant quantity of endotoxin 1.4µg/g, 2.3µg/g, 1.8µg/g, 1.7µg/g and 1.3µg/g respectively in leaves while in square and bolls any cultivar is not able to express significant amount of endotoxin protein to kill insect pests.

When these present cultivars were subjected to combined stresses of high temperature and waterlogging endotoxin expression level was decreased with increase in temperature from 36-40ºC or more from 41-45ºC (Rana *et al*., 2015). When these five cultivars were subjected to both stresses then after 80 DAS endotoxin protein decreases to the extent that no transgenic cultivar was found significant to express more than optimum 1.8µg/g cry protein required to kill insect pests. The lower expression level of Cry protein was alarming as it may cause cross resistance in pests against endotoxin and depriving the glory of Bt technology (Ahmad *et al.,* 2019).

It may specify that raised temperature causes degradation of *Cry1Ac* protein in transgenic cotton cultivars. In contrast with control (25-30ºC and 60-70% humidity) 37ºC temperature and 50% humidity reduces Bt protein content in leaves from 2.6-3% at flowering stage and 3.3- 5.8% at boll filling stage (Zhang *et al*., 2012). In the same manner Chen *et al*., (2012) also resulted that high temperature reduces *Cry1Ac* at flower forming and boll filling phase.

Present results were similar to the previous outcomes of (Yuan *et al*., 2012; Zhang *et al*., 2012; Rana *et al*., 2015) who indicated that raised temperature exerts inverse effects on production of Cry toxin in transgenic cotton. From the above mentioned results *Cry1Ac* gene expression in all cultivars was found highly variable. More than 60% genotypes show 1µg/g *Cry1Ac* protein content in leaves while no cultivar was able to express 1µg/g of endotoxin in flower and bolls at 35-40ºC. Furthermore higher temperature (45ºC) and waterlogging exerts negative impacts on *Cry1Ac* toxin expression in leaves, squares and bolls, with resulting decreased endotoxin level that is reasonably alarming and increases the insect pests attack. Pakistani transgenic cotton harbor only *Cry1Ac* gene and its reduced expression due to high temperature allow the insects to develop resistance. Consequently avoidance to the pest resistance, it is necessary to develop transgenic cotton cultivars that have high expression and comprise of more than one Cry genes.

Conclusion

Combination of abiotic stresses, high temperature and high rainfall, exerted adverse effects on *Cry1Ac* gene expression in transgenic Bt cotton and the fundamental metabolic reactions. Bt protein content in transgenic cotton vary among leaves, squares and bolls within cultivars and between the cultivars grown under normal and stress conditions ultimately leading to high infestation of insect pest.

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