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# Effect of Temperature on *Bt* Gene Expression in Candidate Cotton Lines of Pakistan

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**Abstract:** The expression of *Bt* gene in transgenic cotton is impacted by several abiotic factors. The current study was designed to evaluate the effect of temperature on *Bt* gene expression in selected cotton genotypes under glasshouse condition. A total of 124 genotypes (V-1 to V-124) were cultivated between temperature range 25-30°C. All the genotypes were assessed for the detection of *Bt* gene(s) by immunostrip assay and PCR analysis that revealed the presence of only *Cry1Ac* gene, except for five genotypes. Fresh leaf tissues were collected from all genotypes and *Cry1Ac* gene expression (*Bt* toxin) was quantified through quantitative ELISA kit at 70 days after sowing. Ten genotypes with the highest *Cry1Ac* toxin level (1.00-4.17µg/g) were selected and shifted into 4 different glasshouses under the temperature ranges of 25-30°C, 31-35°C, 36-40°C and 41-45°C respectively. *Cry1Ac* expression level was again quantified after 10 days of varying temperature treatments at 80 days after sowing. Significant reduction in toxin level (P = 0.001) was found under different temperature treatments. The mean toxin level was found to be lowest (1.62µg/g) at the highest temperature range (41-45°C), followed by toxin level (2.01µg/g) at treatment level (36-40 °C). The treatment level (31-35°C) showed higher toxin level (2.50µg/g) as compared to control (25-30°C) (2.29µg/g) and other treatments as well. It can be concluded that mild temperature i.e 31-35°C is the best range for maximum expression *Cry1Ac* toxin in transgenic cotton genotypes. Moreover, higher temperature above 40°C may have adverse effects on the expression of *Cry1Ac* toxin in leaf tissue, with resulting decline in *Bt* toxin level.

**Key words:** *Bt* cotton · *Cry1Ac* · Temperature · Gene Expression · ELISA

## INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is the most important textile fiber crop and the world's 2nd important oil seed crop after soybean. Among top five cotton producing countries, Pakistan is ranked at 4th position [1]. Research over the years has sequentially shown that cotton is susceptible to more than fifteen economically key pests, mainly Lepidopteron insects. In order to improve the range of activity, stability of resistance and protective efficiency, it was focused that variety of genes will be incorporated into plants. To achieve the goal, genetically modified cotton (*Bt* cotton) was engineered by insecticidal genes (*Bt* gene) taken from gram positive, soil bacterium *Bacillus thuringiensis*. *Bt* cotton containing *Cry* gene(s) such as *Cry1Ac*, *Cry1Ac*/*Cry2Ab* or *Cry1Ac*/*Cry1F*, is considerably effective in controlling Lepidopteron pests and is highly beneficial to the grower and the environment by reducing chemical insecticide sprays and preserving population of beneficial arthropods [2].

Transgenic *Bt* cotton is engineered to express δ-endotoxin (*Cry* toxin) proteins in almost all parts of the plant [3]. It has been stated that the mechanism of action

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of the *Bt* (*Cry*) proteins involves the solubilization of crystal in the midgut of insect, proteolytic processing of the protoxin by midgut proteases, binding of the *Cry* toxin to midgut receptors and insertion of the toxin into the apical membrane to create ion channels or pores [4]. When ingested by larvae, toxin proteins bind to particular receptors in the midgut region that disrupts midgut epithelium, thereby causing overall toxic effects and ultimately resulting in death of the larvae [5].

Widespread cultivation of *Bt* cotton in field has shown failure in controlling target insects in few cases [6, 7]. Insect pests control is correlated with the synthesis of insecticidal δ-endotoxin by expression of *Cry* genes [8]. Reduction in toxin level may cause in adequate targeted pest control and provoke resistance in cotton insects against *Cry* proteins [7].The usefulness of genetically modified cotton is allied with the expression level of δ-endotoxin and remains variable throughout the plant life cycle [8, 9]. The efficacy of *Bt* gene against pests also varies among cotton genotypes, plant tissues, various environmental features, types of gene and plant age [10-13].

*Bt* protein content are mainly influenced by abiotic stresses in genetically modified cotton. For example, water logging, salinity, drought, high temperature, high level of $CO_2$ and nitrogen deficiency can significantly reduce the insecticidal protein contents [14-18]. Temperature is one of the important environmental factors affecting the efficiency against bollworm control in *Bt* cotton. *Bt* cotton plants mislay their insect resistance under both low temperature (below 18°C) and high temperature (above 37°C) conditions [6]. It is also examined in recent studies that high temperature causes decrement of *Cry1Ac* protein concentration during the boll filling stage [19, 20].

Genetically modified *Bt* cotton is cultivated in many cotton growing areas of the world for controlling targeted insect pests complex [21], but it is observed that it acts variably in toxin efficiency against target insects under different testing conditions. Cotton belt of Pakistan is in a zone of high temperature (tropical zone) and the summer temperature approaches upto 50°C in some areas and results in heat stress. Heat stress in combination with other environmental stresses such as drought and high light intensity worsen the impact of *Bt* gene efficiency against insect pest complex [22]. Keeping in view the above facts, the present study was designed to evaluate the expression profiling of insecticidal gene in candidate cotton genotype under temperature stress.

## MATERIALS AND METHODS

**Plant Materials and Experimental Design:** During 2012, the seeds of one hundred and twenty four candidate cotton genotypes (V-1 to V-124) were provided by Ayub Agricultural Research Institute, Faisalabad. 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). The plant material was sown in glasshouse (Biosafety Level-II) at National Institute for Genomics and Advanced Biotechnology, 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.

**Temperature Treatments:** All the genotypes were cultivated under normal temperature condition (25-30°C) in glasshouse. Out of 124 genotypes, ten genotypes with the highest expression level were selected at 70 days after sowing and these selected cotton genotypes in triplicates were shifted for another 10 days into 4 different glasshouses under the temperature ranges of 25-30°C, 31-35°C, 36-40°C and 41-45°C, respectively. The temperature was regularly observed in three different glasshouses.

**Polymerase Chain Reaction (PCR):** DNA extraction was done by Cetyl Trimethyl Ammonium Bromide (CTAB) method from the fresh leaf of each entry, according to Doyle and Doyle [23] with slight modifications and optimizations. DNA quantification was done by Nenophotometer™ (IMPLEN Serial No. 1404 UK). From the stock solution samples were diluted to working concentration of 50ng/ìl. The confirmation of *Cry1Ac* (*Bt*) gene was done by event specific primers for Mon-531 event as shown in Table 1. Primers of housekeeping genes for *EF-1-alpha* (*Elongation factor 1-alpha*) and *Sad1* (*stearoyl-ACP desaturase*) were also synthesized and used in reaction to confirm the validity of working conditions for PCR.

PCR was carried out using Veriti® thermal cycler (Applied Biosystems, USA). Reaction mixture having; 10X Taq Buffer with $(NH_4)_2SO_4$, 25mM $MgCl_2$, 5U/ìlTaq DNA polymerase, 10mM dNTP Mix, both forward and reverse primers 20pM, DNA 50ng/ìl and doubled distilled $H_2O$ was used in each PCR reaction. The reaction conditions were

Table 1: Sets of primers used in Polymerase Chain Reaction (PCR)

| Target | Primer Name | Sequences (5′ ------ 3′) | Specificity | Amplicon | Reference |
|---|---|---|---|---|---|
| Mon-531 | Mon-531 F | AAGAGAAACCCCAATCATAAAA | Genome/*Cry1Ac* | 346bp | [24]. |
| | Mon-531R | GAGAATGCGGTAAAGATACGTC | | | |
| Cotton endogenous | *EF-1á* F | TTCGAGAAGGAAGCTGCTGA | *Elongation factor 1-alpha* | 172bp | This Paper |
| | *EF-1á* R | TAAAGTCTCTGTGTCCGGGG | | | |
| Cotton endogenous | *Sad1* F | CCAAAGGAGGTGCCTGTTCA | *Stearoyl-ACP desaturase* | 107bp | [24]. |
| | *Sad1* R | TTGAGGTGAGTCAGAATGTTGTTC | | | |

optimized at: a preheat temperature of 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; and a final elongation at 72°C for 10 min.

The PCR product was separated on 1% agarose gel (Pelican Life Sciences, Company). Amplified product was visualized by UV illuminator at 260nm wavelength and photographed through gel documentation system. The PCR products obtained were compared with 100bp ladders (Fermentas).

**Immunostrip Assay:** Approximately 100mg fresh leaf tissue of each genotype was collected for immunostrip assay for detection of *Bt* protein(s). Samples were prepared as per manufacturer's instructions (Agdia Inc. USA) and were tested for the detection of *Cry* protein(s) *Cry1Ac*, *Cry2Ab* and *Cry1F*. Strips 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. Results of the reactions were recorded as positive (+) and negative (-) on the bases of test line appearance on the strip.

**Sandwich-Enzyme Linked Immunosorbent Assay (ELISA):** All the genotypes/entries that showed positive reactions for the presence of *Cry1Ac* gene with immunostrip assay were further exposed to sandwich ELISA for quantification of *Cry1Ac* protein (*Bt* toxin) at 70 days after sowing (DAS).Third fully expanded leaf tissues of the all the plant entries/genotypes (124) grown in normal temperature range/treatment (25-30°C) were used for analysis. Twenty milligram of fresh plant tissues were collected, ground manually in extraction buffer provided by manufacturer of kit and ELISA was performed for the quantification of *Bt* toxin level following the instructions of manufacturer (Envirologix Inc. USA).

Leaf samples of the 10 selected cotton genotypes grown under three different temperature treatments (25-30°C, 31-35°C, 36-40°C and 41-45°C) were also analyzed in similar way at 80 DAS. To calculate the toxin quantity in each sample, optical density was measured by Microplate Reader (BIORADiMark™) at wavelength of 450 nm. Finally, toxin levels were calculated as μg/g by using simple regression analysis.

**Statistical Analysis:** Two way analysis of variance (ANOVA) was applied to analyze toxin levels under different temperature stress levels and their means were compared using least significant difference. Statistix v. 8.1 [25] package was used for this purpose.

## RESULTS

**Detection of *Cry* protein(s):** One hundred and twenty four cotton genotypes were tested by immunostrip assay for the detection of *Bt* genes viz., *Cry1Ac*, *Cry2Ab* and *Cry1F*. Result showed that all the genotypes carried *Cry1Ac* gene except for V-59, V-71, V-72, V121 and V-122 as shown in Table 2. All the genotypes showed 100% negative reactions by immunostrip assay for *Cry2Ab* (Bollgard-II event) and *Cry1F* (Wide Strike event) genes.

**Confirmation of *Cry1Ac* gene (Mon531 Event):** PCR result confirmed the presence of Mon-531 event in all of the cotton genotypes except for V-59, V-71, V-72, V121 and V-122. PCR product of 346bp was amplified in all Mon-531 positive genotypes (Fig. 1& 2). Along with Mon-531 cotton endogenous gene *Sad1* was also amplified in genotypes from V-1 to V-69 with the product size of 107bp.While from V-70 onward *EF-1-alpha* (Product 172bp) was amplified with Mon-531.

**Quantification of *Cry1Ac* protein (*Bt* Toxin):** Leaf samples were taken from individual plants under normal temperature condition (25-30°C) and toxin level was quantified through sandwich ELISA at 70 DAS. The results of *Cry1Ac* toxin level for all the genotypes (V-1 toV-124) at normal temperature range (25-30°C) are shown in Table 3. 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 0.026- 4.286μg/g.

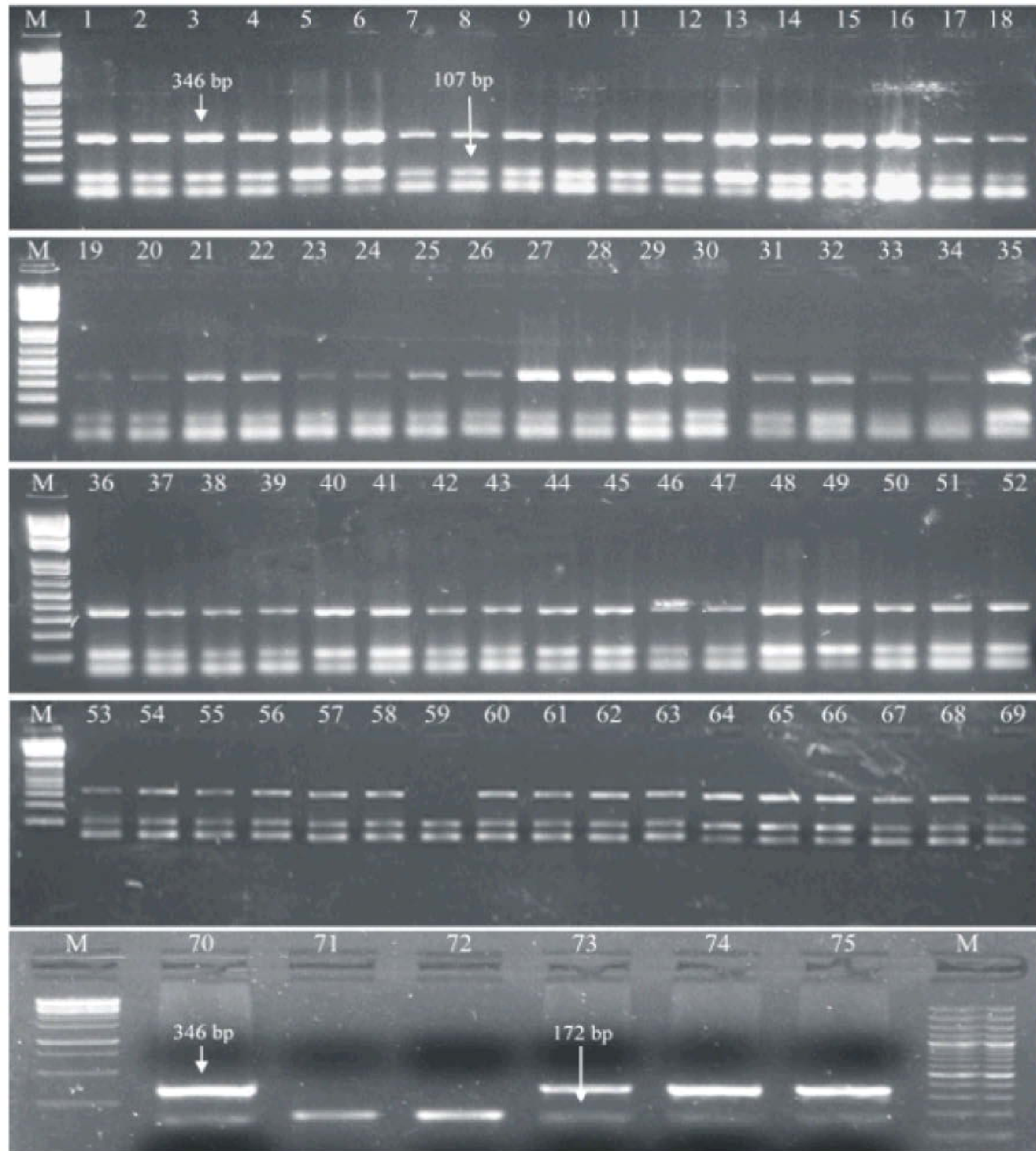


Fig. 1: Amplification of Mon-531 event in 75 genotypes through PCR: 1-69 is the amplification of Mon-531 (346bp) with *Sad1* (107 bp) and from 70-75 is the amplification of Mon-531 (346bp) with *EF-1-alpha* (172bp). 59, 71 and 72 are negative for Mon-531. 100bp DNA ladder is used (Fermentas)

Five genotypes (V-59, V-71, V-72, V-121 and V-122) were found to be negative for *Cry1Ac* toxin expression. Among all the genotypes, V-120, V-98, V-61, V-113 gave the minimum expression level (0.026, 0.041, 0.049 and 0.050µg/g, respectively) while10 out of 124 genotypes (V-76, V-77, V-81, V-93, V-96, V-102, V-106, V-112, V-114 and V-118) expressed the maximum level of *Cry1Ac* toxin ranged from 1.00-4.286µg/g at 25-30°C.

The level of *Cry1Ac* toxin in ten selected cotton genotypes was also quantified at 80 DAS under varying temperature conditions/treatments (25-30°C, 31-35°C, 36-40°C and 41-45°C) in different glass houses as shown in Table 4. Both temperature treatments and genotypes were found to have significant effects (F = 173.37; P = 0.001 and F = 503.27; P = 0.001) on *Cry1Ac* toxin level. The overall mean toxin level was found to be lowest

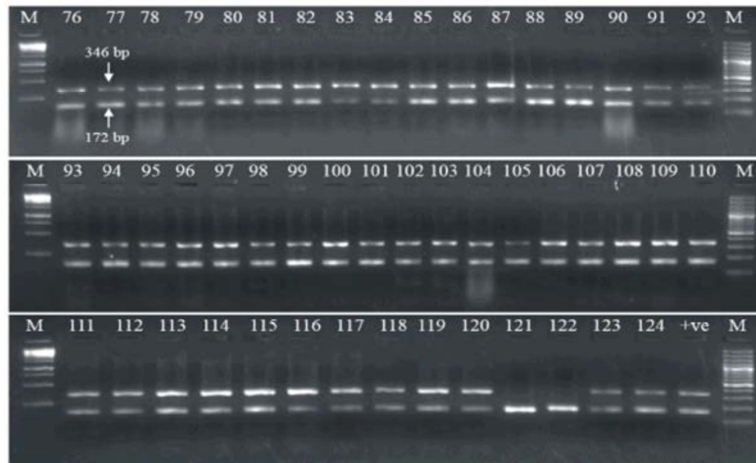


Fig. 2: Amplification of Mon-531 event in 49 genotypes through PCR: 76-124 amplification of Mon-531 (346bp) with *EF-1-alpha* (172bp). 121 and 122 are negative for Mon-531. 1kbp and 100bp DNA ladders were used (Fermentas).

Table 2: Results for Immunostrip assay of 124 candidate cotton genotypes

| Genotype | Cry1Ac | Cry2Ab/1F | Genotype | Cry1Ac | Cry2Ab/1F | Genotype | Cry1Ac | Cry2Ab/1F |
|---|---|---|---|---|---|---|---|---|
| V-1 | +ve | -ve | V-43 | +ve | -ve | V-85 | +ve | -ve |
| V-2 | +ve | -ve | V-44 | +ve | -ve | V-86 | +ve | -ve |
| V-3 | +ve | -ve | V-45 | +ve | -ve | V-87 | +ve | -ve |
| V-4 | +ve | -ve | V-46 | +ve | -ve | V-88 | +ve | -ve |
| V-5 | +ve | -ve | V-47 | +ve | -ve | V-89 | +ve | -ve |
| V-6 | +ve | -ve | V-48 | +ve | -ve | V-90 | +ve | -ve |
| V-7 | +ve | -ve | V-49 | +ve | -ve | V-91 | +ve | -ve |
| V-8 | +ve | -ve | V-50 | +ve | -ve | V-92 | +ve | -ve |
| V-9 | +ve | -ve | V-51 | +ve | -ve | V-93 | +ve | -ve |
| V-10 | +ve | -ve | V-52 | +ve | -ve | V-94 | +ve | -ve |
| V-11 | +ve | -ve | V-53 | +ve | -ve | V-95 | +ve | -ve |
| V-12 | +ve | -ve | V-54 | +ve | -ve | V-96 | +ve | -ve |
| V-13 | +ve | -ve | V-55 | +ve | -ve | V-97 | +ve | -ve |
| V-14 | +ve | -ve | V-56 | +ve | -ve | V-98 | +ve | -ve |
| V-15 | +ve | -ve | V-57 | +ve | -ve | V-99 | +ve | -ve |
| V-16 | +ve | -ve | V-58 | +ve | -ve | V-100 | +ve | -ve |
| V-17 | +ve | -ve | V-59 | -ve | -ve | V-101 | +ve | -ve |
| V-18 | +ve | -ve | V-60 | +ve | -ve | V-102 | +ve | -ve |
| V-19 | +ve | -ve | V-61 | +ve | -ve | V-103 | +ve | -ve |
| V-20 | +ve | -ve | V-62 | +ve | -ve | V-104 | +ve | -ve |
| V-21 | +ve | -ve | V-63 | +ve | -ve | V-105 | +ve | -ve |
| V-22 | +ve | -ve | V-64 | +ve | -ve | V-106 | +ve | -ve |
| V-23 | +ve | -ve | V-65 | +ve | -ve | V-107 | +ve | -ve |
| V-24 | +ve | -ve | V-66 | +ve | -ve | V-108 | +ve | -ve |
| V-25 | +ve | -ve | V-67 | +ve | -ve | V-109 | +ve | -ve |
| V-26 | +ve | -ve | V-68 | +ve | -ve | V-110 | +ve | -ve |
| V-27 | +ve | -ve | V-69 | +ve | -ve | V-111 | +ve | -ve |
| V-28 | +ve | -ve | V-70 | +ve | -ve | V-112 | +ve | -ve |
| V-29 | +ve | -ve | V-71 | -ve | -ve | V-113 | +ve | -ve |
| V-30 | +ve | -ve | V-72 | -ve | -ve | V-114 | +ve | -ve |
| V-31 | +ve | -ve | V-73 | +ve | -ve | V-115 | +ve | -ve |
| V-32 | +ve | -ve | V-74 | +ve | -ve | V-116 | +ve | -ve |
| V-33 | +ve | -ve | V-75 | +ve | -ve | V-117 | +ve | -ve |
| V-34 | +ve | -ve | V-76 | +ve | -ve | V-118 | +ve | -ve |
| V-35 | +ve | -ve | V-77 | +ve | -ve | V-119 | +ve | -ve |
| V-36 | +ve | -ve | V-78 | +ve | -ve | V-120 | +ve | -ve |
| V-37 | +ve | -ve | V-79 | +ve | -ve | V-121 | -ve | -ve |
| V-38 | +ve | -ve | V-80 | +ve | -ve | V-122 | -ve | -ve |
| V-39 | +ve | -ve | V-81 | +ve | -ve | V-123 | +ve | -ve |
| V-40 | +ve | -ve | V-82 | +ve | -ve | V-124 | +ve | -ve |
| V-41 | +ve | -ve | V-83 | +ve | -ve | ….. | ….. | ….. |
| V-42 | +ve | -ve | V-84 | +ve | -ve | ….. | ….. | ….. |

+ve = *Bt* gene presence; -ve = *Bt* gene absence; V-59, V-71, V72, V-121 and V-122 are negative with Immunostrips assay for *Cry1Ac* protein.

Table 3: Quantification of *Cry1Ac* protein (*Bt* toxin) expression for all (V-1 to V-124) genotypes at 70 DAS (days after sowing) under normal temperature conditions (25-30°C)

| Genotypes | *Cry1Ac* toxin (µg/g) | Genotypes | *Cry1Ac* toxin (µg/g) | Genotypes | *Cry1Ac* toxin (µg/g) | Genotypes | *Cry1Ac* toxin (µg/g) |
|---|---|---|---|---|---|---|---|
| V-1 | 0.633 | V-32 | 0.444 | V-63 | 0.071 | V-94 | 0.266 |
| V-2 | 0.672 | V-33 | 0.356 | V-64 | 0.067 | V-95 | 0.430 |
| V-3 | 0.517 | V-34 | 0.305 | V-65 | 0.720 | V-96 | 4.286 |
| V-4 | 0.478 | V-35 | 0.393 | V-66 | 0.584 | V-97 | 0.146 |
| V-5 | 0.567 | V-36 | 0.419 | V-67 | 0.569 | V-98 | 0.041 |
| V-6 | 0.475 | V-37 | 0.648 | V-68 | 0.609 | V-99 | 0.092 |
| V-7 | 0.543 | V-38 | 0.415 | V-69 | 0.773 | V-100 | 0.203 |
| V-8 | 0.579 | V-39 | 0.610 | V-70 | 0.071 | V-101 | 0.227 |
| V-9 | 0.800 | V-40 | 0.627 | V-71 | 0.00 | V-102 | 3.251 |
| V-10 | 0.703 | V-41 | 0.368 | V-72 | 0.00 | V-103 | 0.855 |
| V-11 | 0.760 | V-42 | 0.602 | V-73 | 0.094 | V-104 | 0.245 |
| V-12 | 0.318 | V-43 | 0.500 | V-74 | 0.281 | V-105 | 0.162 |
| V-13 | 0.332 | V-44 | 0.616 | V-75 | 0.587 | V-106 | 2.386 |
| V-14 | 0.530 | V-45 | 0.725 | V-76 | 2.137 | V-107 | 0.898 |
| V-15 | 0.786 | V-46 | 0.459 | V-77 | 1.119 | V-108 | 0.749 |
| V-16 | 0.461 | V-47 | 0.385 | V-78 | 0.179 | V-109 | 0.200 |
| V-17 | 0.822 | V-48 | 0.813 | V-79 | 0.979 | V-110 | 0.976 |
| V-18 | 0.455 | V-49 | 0.330 | V-80 | 0.651 | V-111 | 0.075 |
| V-19 | 0.729 | V-50 | 0.392 | V-81 | 1.636 | V-112 | 2.401 |
| V-20 | 0.389 | V-51 | 0.638 | V-82 | 0.408 | V-113 | 0.050 |
| V-21 | 0.621 | V-52 | 0.476 | V-83 | 0.491 | V-114 | 1.455 |
| V-22 | 0.861 | V-53 | 0.507 | V-84 | 0.114 | V-115 | 0.138 |
| V-23 | 0.412 | V-54 | 0.626 | V-85 | 0.508 | V-116 | 1.557 |
| V-24 | 0.697 | V-55 | 0.224 | V-86 | 0.784 | V-117 | 0.209 |
| V-25 | 0.676 | V-56 | 0.385 | V-87 | 0.585 | V-118 | 1.249 |
| V-26 | 0.462 | V-57 | 0.242 | V-88 | 0.240 | V-119 | 0.133 |
| V-27 | 0.717 | V-58 | 0.067 | V-89 | 0.654 | V-120 | 0.026 |
| V-28 | 0.533 | V-59 | 0.00 | V-90 | 0.602 | V-121 | 0.00 |
| V-29 | 0.583 | V-60 | 0.095 | V-91 | 0.281 | V-122 | 0.00 |
| V-30 | 0.286 | V-61 | 0.049 | V-92 | 0.148 | V-123 | 0.110 |
| V-31 | 0.081 | V-62 | 0.761 | V-93 | 4.031 | V-124 | 0.389 |

V-59, V-71, V72, V-121 and V-122 are with 0.00 µg/g of *Cry1Ac* protein (*Bt* toxin): 0.00 shows no expression level of *Cry1Ac* toxin (-ve).

Table 4: Cry1Ac toxin expression level for the selected genotypes at varying temperature conditions (25-30°C, 31-35°C, 36-40°C and 41-45°C) at 80 DAS

| | Temperature treatments | | | | |
|---|---|---|---|---|---|
| Genotypes | 25-30°C | 31-35°C | 36-40°C | 41-45°C | Mean |
| V-76 | $2.06^{jk}$ | $2.1^{i\text{-}k}$ | $2.00^{kl}$ | $1.92^{kl}$ | $2.02^{e}$ |
| V-77 | $1.00^{r\text{-}t}$ | $1.02^{rs}$ | $0.97^{st}$ | $0.75^{tu}$ | $0.94^{g}$ |
| V-81 | $1.33^{o\text{-}q}$ | $1.49^{n\text{-}p}$ | $1.12^{q\text{-}s}$ | $0.94^{st}$ | $1.22^{f}$ |
| V-93 | $3.96^{b}$ | $4.06^{ab}$ | $3.02^{de}$ | $2.47^{gh}$ | $3.38^{b}$ |
| V-96 | $4.17^{ab}$ | $4.30^{a}$ | $3.96^{b}$ | $3.07^{de}$ | $3.88^{a}$ |
| V-102 | $3.18^{cd}$ | $3.36^{c}$ | $2.85^{ef}$ | $2.11^{i\text{-}j}$ | $2.88^{c}$ |
| V-106 | $2.27^{h\text{-}j}$ | $3.00^{de}$ | $1.78^{lm}$ | $1.52^{m\text{-}o}$ | $2.14^{e}$ |
| V-112 | $2.34^{hi}$ | $2.65^{fg}$ | $2.31^{h\text{-}j}$ | $1.93^{kl}$ | $2.31^{d}$ |
| V-114 | $1.34^{o\text{-}q}$ | $1.65^{mn}$ | $1.02^{rs}$ | $0.91^{st}$ | $1.23^{f}$ |
| V-116 | $1.25^{p\text{-}r}$ | $1.36^{o\text{-}q}$ | $1.02^{rs}$ | $0.61^{u}$ | $1.06^{g}$ |
| Mean | $2.29^{b}$ | $2.50^{a}$ | $2.01^{c}$ | $1.62^{d}$ | |

Means followed by the same letters within the column are not significantly different ($p>0.05$; LSD)

$LSD_{(0.05)}$ for Genotypes = 0.131

$LSD_{(0.05)}$ for Treatments = 0.082

$LSD_{(0.05)}$ for Genotypes x Treatments = 0.261

(1.62 µg/g) at temperature range (41-45 $^0$C), followed by toxin level (2.01 µg/g) at treatment level (36-40 °C). At (31-35°C) higher toxin level (2.50µg/g) was found as compared to control (25-30°C) (2.29µg/g) as well as other treatments (Table 4). Regarding genotypes, V-96 showed highest mean toxin level 3.88µg/g, followed by V-93 (3.38µg/g) and V-102 (2.88µg/g). While the lowest mean toxin level (0.94µg/g) was found in the genotype V-77 in ten selected cotton genotypes (Table 4).

## DISCUSSION

In the present study *Bt* genes (*Cry1Ac; Cry1F* and *Cry2Ab*) were first confirmed through immunostrip assay and it has been verified that only *Cry1Ac* gene exists in all the tested cotton genotypes except for five genotypes. According to the finding of Yang *et al*. [24] Mon-531 event is specific for *Cry1Ac* gene only. PCR results of present study confirmed the presence of only Mon-531 event in all genotypes except for five genotypes. Ali *et al*. [26] also confirmed the presence of only *Cry1Ac* gene through molecular analysis. Molecular analysis revealed the presence of Mon-531 event of Monsanto in Pakistani genotypes with the absence of any other patented events (Mon-1445 and Mon-15875).

For protection against specific insect pests, appropriate level of toxin is important at specific stage and time. According to Kranthi *et al*. [5] for durable pest resistance *Bt* toxin 1.8µg/g fresh weight basis or higher is recommended. Among the ten selected genotypes, V-76, V-93, V-96, V-102 V-106, V-112 showed the highest mean toxin level (2.02, 3.38, 3.88, 2.88, 2.14 and 2.31µg/g, respectively) under all the temperature treatments at 80 DAS. In all the selected genotypes, toxin expression level was decreased with further increase in temperature (36-40°C and 41-45°C) indicating that high temperature may cause the degradation of *Cry1Ac* protein in transgenic cotton genotypes.

In present study plant genetic backgrounds have been found to effect the *Bt* gene expression. Except for five genotypes (V-76, V-93, V-96, V-102, V-106 and V-112) the *Cry1Ac* expression level in most of tested genotypes was found lower than 1.8 µg/g fresh weight basis at 80 DAS. This low level of expression is alarming as it may provoke cross resistance in insect against *Cry1Ac*, depriving the farmers to the benefit of *Bt* technology. Zhang *et al*. [20] analyzed that high temperature (37°C) is the major cause in the decrement of *Bt* toxin level. According to them, in comparison to the control (25-30°C and 60%-70% humidity), 37°C and 50% humidity decrease leaf *Bt* protein contents 3.3% to 5.8% at peak boll formation stage and 2.6% to 3.0% at peak flowering stage. Chen *et al*. [19] also concluded that *Cry1Ac* protein expression in leaves is significantly affected by extreme temperature at peak flowering and boll filling stage. The results of present study are closely related to the findings of Chen *et al*. [19] and Zhang *et al*. [20] who revealed that high temperature stress in inversely proportional to the *Cy1Ac* expression level.

From the results it is concluded that in all tested genotypes the expression of *Cry1Ac* is highly variable. More than 92% of selected genotypes expressed toxin less than 1µg/g of fresh weight at control temperature range (25-30°C). The temperature range i.e. 31-35°C is found to be the optimum range for *Bt* toxin expression in Pakistani cotton genotypes. Moreover, higher temperature (above 40°C) may have negative effects on *Cry1Ac* toxin expression of in leaf tissue, with resulting decline in *Bt* toxin level expression that is quite alarming because temperature stress condition may increase the insect pests attack. So to delay the risk of resistance development in pests, genotypes having low *Cry1Ac* toxin level should not be cultivated. As Pakistani cotton genotypes harbor only *Cry1Ac* gene, the packages of *Bt* genes may be a solution to delay and avoid the risk of development of cross resistance in target cotton insect/pests.

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