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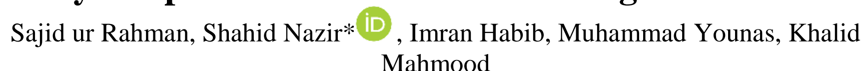
Effects of temperature stresses and growth stages on Cry1Ac protein accumulation in Bollgard^{TM} cotton



Sajid ur Rahman, Shahid Nazir*, Imran Habib, Muhammad Younas, Khalid Mahmood

Abstract

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium that makes crystal proteins toxic for insects, particularly larvae of Diptera and Lepidopteron. Bt genes has been frequently used to develop genetically modified (GM) insect resistant crops. Various factors affecting the gene expression and protein accumulation in GM crops hence, present work was designed to evaluate the effects of temperatures and age on expression pattern of *Cry1Ac* gene in Bt cotton. Transformation event was verified using digital PCR and the presence of endotoxin was initially confirmed through immuno-strip assay. Toxin accumulation level in top third leaf was investigated using quantitative ELISA after 20 days intervals starting from 60 days. Results showed that protein level gradually increased with plant's age, and it was maximum at 100 days in plants placed under 35°C. Toxin level declined under all temperature regimes at advanced growth stages and minimal toxin was observed at 25°C. Bt cotton MNH-886 showed higher and stable toxin accumulation in comparison with FH-lalazar even at elevated temperatures and lateral growth stages. These findings demonstrated that expression of *Cry1Ac* gene is influenced by temperatures and age of the plant, while MNH-886 is a more stable genotype regarding Cry1Ac protein accumulation.

Keywords: Bt Cotton, Temperatures, Growth stages, ELISA
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Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute, Faisalabad;
*Corresponding Author's Email: shahidnazir97@yahoo.com

Introduction

Cotton (*Gossypium hirsutum* L.) is Pakistan's most important cash crop and is a main source for textile fiber. Local edible oil production and animal feed is also supplemented from cotton. Pakistan is ranked as the 5th leading cotton producer after China, India, USA and Brazil (Shahbandeh, 2021). It contributes 3.1 % of value-added in agriculture and around 0.6% to Gross Domestic Products (GDP). During 2020-21, cotton crop was cultivated on an area of 2079 thousand hectares, with production of 7064 thousand bales (Anonymous, 2020-2021). Cotton production is affected by various insects/pests, diseases, and environmental factors. Among these, insects are the major problems being faced by cotton growers, and different insecticides/pesticides are used to minimize yield losses. There are two major types of insects; chewing and sucking. All bollworms come under chewing types of insets and are major threat to cotton in Pakistan. Different insecticides/pesticides have been used for the last four decades, which is an extra burden not only on our farmers but our economy and environment also affected very badly. Hence, there is a need to develop and adopt such technologies that can minimize the use of these toxic chemicals and clean the environment. Several conventional approaches are being underway to develop insect-resistant cotton, but the introduction of genetically modified (GM) plants harboring insect resistant transgene(s) has proved to be a best approach (Ali, 2010). *Bacillus thuringiensis* (Bt*)* is an aerobic, gram-positive, ubiquitous and soil-dwelling bacterium having crystalline (cry) proteins in cytoplasm of its sporulating cells. These cry proteins are highly poisonous even at low concentrations to some sucking and chewing insects of dipteran and lepidopteran orders (Schnepf et al., 1998). There are about eighty different bacterial types capable to produce a large amount of various toxins e.g. exotoxins, enterotoxins and endotoxins. These Bt toxic protein are primarily divided into two main groups i.e. Cytolytic (Cyt) and Crystal (Cry). The cotton crop has been transgenically modified and its production also increased significantly by introducing the responsible genes for toxic proteins from bacteria. These Bt proteins not only control the attack of chewing and sucking insects on cotton but at the same time it also protects some beneficial arthropods (Tabashnik et al., 2002). When insect larvae chew the toxic protein, it binds to specific receptors presents in the midgut of larvae and causes to disrupt the epithelium and ultimately leads to larval death (Betz et al., 2000). These toxic proteins are highly precise in actions and are very specific to insects. Applications of pesticides on cotton to control the insects has been decreased from an average of 12-13 sprays on conventional genotypes to 3-4 sprays on GM varieties (Ali, 2013). Cotton crop is very sensitive at various growth stages and requires an elevated amount of toxic protein for an efficient insect control. Because, there are many environmental and internal factors affects the transgene(s) and their expression also variable at different growth periods (Kranthi et al., 2005; Khan et al., 2017). Effects of soil, nutrition, temperature, CO_2, etc. are also found to be important factors for the expression of Bt genes and accumulation of toxic proteins in cotton (Adamczyk & Sumerford, 2001; Chen et al., 2005). It is assumed that the changed efficiency of resistance against insects might be linked with high temperature and age of plant. In Pakistan, cotton having *Cry1Ac* gene of Mon531 event (Bollgard™ cotton) is the only GM crop officially approved for commercial cultivation. Keeping in view the

importance of temperatures and plant's age, present study was designed to find the accumulation level of toxic protein in Bollgard™ cotton at different growth stages and with variable temperature stresses. The variability in toxin accumulation was estimated in top third leaf of two high-yielding and mostly adopted Bt-cotton genotypes using ELISA technique.

Materials and methods

Plant material and growth conditions

The pure seeds of two high-yielding and widely adopted Bt cotton varieties, i.e., MNH-886 and FH-lalazar (local origin) were delinted with concentrated sulphuric acid (H_2SO_4). Seeds were carefully washed under tap water and floating seeds were discarded before sowing in polythene bags having a mixture of sandy loam soil and farmyard manure @ 3:1 ratio respectively. Equally geminated plantlets were shifted into earthen pots of 38cm diameter and 40cm height having same soil composition. When 3-5 true leaves were developed, weak plantlets were thinned out and a single healthy plant was allowed to grow in each pot. A total of 50 pots for each variety were maintained for further studies.

Variable temperature stresses

Four different temperature treatments were applied to 40 days old plants under controlled glasshouse conditions, i.e. (i) 25±3°C, (ii) 30±3°C (iii) 35±3°C and (iv) 40±3°C. Air conditioners and electric heaters were used to maintain the defined temperature conditions. The potted plants were watered at regular intervals to provide proper moisture conditions and necessary agronomic practices were also continued for healthy growth and development.

Genomic DNA isolation and molecular verification of Bollgard™ cotton

Total genomic DNA was isolated from leaves using CTAB method (Sambrook & Russel, 2001). Quantification of DNA was carried out using Non-drop spectrophotometer (ND-2000) and 30ng of isolated DNA was used in PCR to confirm the Bollgard™ cotton using Mon531 event-specific primers as described by Yang *et al*., 2005. The master mix for PCR analysis was comprised on: 2.0µl of 10X PCR buffer, 1.5µl of 25mM $MgCl_2$, 1.5µl of 2.0mM dNTPs, 1.0µl of 1.0µM each of forward and reverse primers, 0.2µl of 5U/µl Taq DNA polymerase, 2.0µl of 30ng/µl of isolated DNA and 10.8µl of d_3H_2O to make the final volume up to 20µl. Thermal cycler profile was consisted of 35 cycles with denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and initial extension at 72°C for 45 sec with one final extension step at 72°C for 7min. PCR amplification results were visualized using gel documentation system equipped with Ultra Violet light (Photonyx Ultra, NYX Tecnhnik USA).

Qualitative analysis for toxic cry protein

The presence of toxin was determined using Cry1Ac protein specific strips (Envirologix Inc. USA). Fresh leaf sample (20mg) from both Bt cotton (MNH-886, FH-lalazar) and a non-Bt was ground in 500µl of protein extraction PBS buffer in 2.0ml centrifuge tubes. Strips were placed in each tube and left for 5-10 minutes at room temperature. The results appeared in the form of test lines which were noted as positive (+) for presence and negative (-) for absence of toxin in tested cotton varieties.

Quantitative analysis for toxic cry protein

Enzymes Linked Immuno-Sorbent Assay (ELISA) was performed for the quantitative assessment of cry protein accumulation level when plant reached at the age of 60, 80, 100 and 120 days. Fresh leaf sample (20mg) from both Bt cotton varieties was ground in protein extraction buffer according to manufacturer's instructions (Envirologix Inc. USA). Samples were loaded into antigen pre coated plates and analyzed under ELISA plate reader (uQuant BioTek, USA). Toxin accumulation level was analyzed and calculated using Gen-5 software.

Statistical analysis of experimental data

Data was subjected to analysis of variance (ANOVA) using Statistics 8.1 and Microsoft Excel 2010. The effects of variable temperature treatments, growth stages, varieties and their interactions with toxin levels were evaluated. Mean and standard deviations were descriptive measures of quantitative data using the analysis of variance for independent samples. P-values of $P \leq 0.05$ were considered significant.

Results

Duplex PCR to verify bollgard™ transformation event

Equal quantities of isolated DNA from both Bt cotton varieties was used as template in PCR while DNA from non-Bt cotton also used as a negative control for *Cry1Ac* gene. Bollgard™ event specific primers were used to confirm the transformation event. A second primer set of housekeeping gene for cotton was also included to make a duplex PCR reaction and to verify the cotton DNA (Table-1). Similar PCR amplification profile was optimized for both genes. Amplicons were separated on 1.5% agarose gel. Figure-1A showing successful amplification of 183bp of housekeeping gene from all samples and confirmed the presence of cotton DNA in reaction mixture. Another fragment of 346bp amplified from two cotton varieties while this fragment was not present in non-Bt cotton sample. No PCR amplification was observed in PCR negative control sample which has d_3H_2O as template. This negative control sample also verify that there is no contamination in consumables. These molecular analysis verifying that both varieties i.e. MNH-886 and FH-lalazar have same *Cry1Ac* gene of Mon531 event of bollgard™ cotton.

Table 1. Primer sequences used for molecular confirmation of transformation event

Gene / event name	Primer sequence	Size
Mon531	Forward: AAG AGA AAC CCC AAT CAT AAA A Reverse: GAG AAT GCG GTA AAG ATA CGT C	346bp
CIR183	Forward: CCA GAT TAG AAC CTA TGA AAC Reverse: TAG CCC ATT TCT TAC CAC	183bp

Strip test for preliminary assessment of toxic cry protein

PCR positive plants were qualitatively analyzed for the presence of toxin using cry protein specific strips. This protein identification assay is very easy, fast and cheap as it does not require any sophisticated tool. Cry1Ac protein specific strips were placed in ground leaf samples and allowed to complete the reaction. It was observed that buffer started to move upward direction of strip through capillary action. One sample from non-Bt cotton was also used as negative control for toxic protein and to justify the results.

With the movement of buffer, one control line was appeared on all strips which was showing that whole experimental procedure is satisfactory and reliable. The second line specific for Cy1Ac protein only appeared on those strips which were placed in PCR positive Bt cotton samples (Figure-1B). This assay indicated that the insect resistant *Cry1Ac* gene is fully functional and translating itself into desired cry protein in leaf tissues. This qualitative assay revealed that both targeted Bt cotton varieties accumulating the toxic cry protein but its level cannot be measured with strip test.

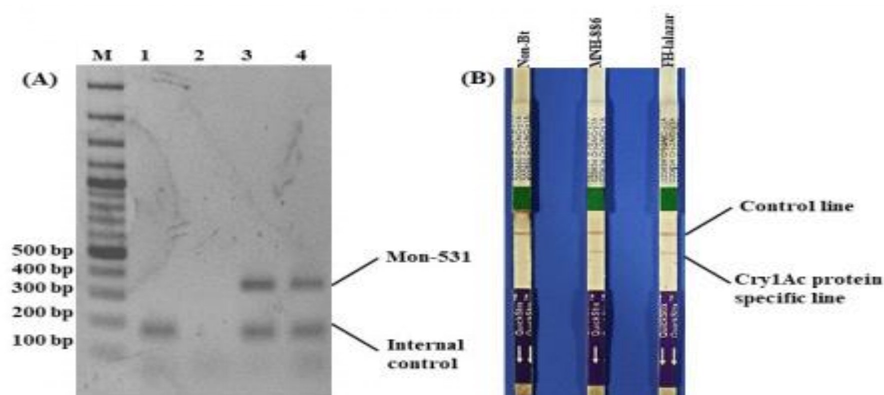

Figure 1. (A) Duplex PCR for the confirmation of Mon531 event using event-specific and cotton internal control primers. Lane **M:** 100 bp DNA marker, lane **1:** DNA from Non-Bt cotton, lane **2:** PCR –ve control without any template DNA, lane **3:** DNA from FH-lalazar, lane **4:** DNA from MNH-886. **(B)** Strip test to detect Cry1Ac protein in leaf tissues of Bt cotton plants.

Assessment of toxin level in leaves at 60 days

Bt plants that showed positive reactions in qualitative strip assay were further quantitatively analyzed to verify the accumulation level of toxic protein using Enzyme Linked Immuno-Sorbent Assay (ELISA). When the plants reached at the age of 60 days, leaves were taken from individual plants maintained at four different temperatures, and toxin level was quantified. The results showed that the accumulation level of toxic protein increased upto specific limit and after that it tends to decline with increase in temperature (Figure-2). Plants placed at 35°C showed higher toxin level, i.e. 1.31µg/g on fresh leaf weight basis, whereas this level was decreased to 1.11 µg/g at 40°C. Protein level at 30°C was nearly similar to 35°C. At this stage, minimum amount of endotoxin was observed at lower temperature i.e. 25°C and both cotton genotypes showed almost similar toxin levels with 0.64µg/g and 0.66µg/g in FH-lalazar and MNH-886 respectively.

Assessment of toxin level in leaf at 80 days

Toxic protein was quantified in leaf samples taken from individual Bt cotton plants grown at different temperature conditions at the age of 80 days. Results showed that the level of Cry1Ac in leaf tissues at 30°C was 1.53µg/g. While after increasing temperature,

toxin levels in leaf tissues were observed to increase i.e. 1.66µg/g at 35°C. However, with further rise in temperature, accumulation level was started to decrease in both cotton varieties i.e. 1.32µg/g in MNH-886 and 1.19µg/g in FH-lalazar at 40°C. On other side, at low temperature, lower toxin accumulation level was observed (0.91µg/g). Significant differences also found among both Bt cotton varieties at this growth stage. MNH-886 accumulates higher levels of cry toxin in comparison with FH-lalazar. Results showed that both Bt cotton varieties vary from one another with reference to toxin accumulation levels at 80 days (Figure-2).

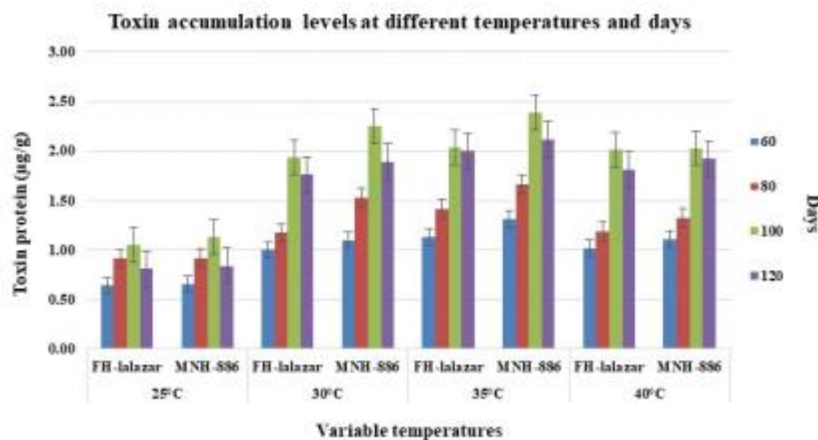

Figure 2. Accumulation of Cry1Ac toxic protein in leaves of two bollgard™ cotton varieties under variable temperature stresses and at different growth stages.

Assessment of toxin level in leaf at 100 days

Accumulation of cry endotoxin was verified using quantitative ELISA method in top third leaf of both cotton varieties at 100 days placed at four variable temperatures. Data revealed that average accumulation of toxic protein at this stage was 1.87µg/g. The highest level of toxin accumulation was observed in MHN-886 with 2.39µg/g at 35°C, while FH-lalazar accumulated 2.04µg/g at similar conditions (Figure-2). A comparable pattern of decline in toxin accumulation was also observed at this stage with the increase in temperature. In contrast, lower accumulation level was detected in leaf tissue allowed to develop at 25°C, which accumulated 1.18µg/g and 1.93µg/g of toxin in FH-lalazar and MNH-886 respectively. This growth stage also showed significant differences in accumulation levels of cry toxin in both cotton varieties. These results showed that the expression of *Cry1Ac* gene was higher in both varieties at 100 days and cry toxin accumulation level was also significantly increased in MNH-886.

Assessment of toxin level in leaf at 120 days

Significant differences were observed in crystal protein accumulation at the age of 120 days. Results indicated that toxin level was starting to decline in both genotypes at all temperatures. The average accumulation level of toxic protein was decreased from

1.87µg/g at 100 days to 1.73µg/g at 120 days in leaf tissues (Figure-2). At high temperature i.e. 40°C, the average protein level in FH-lalazar was decreased from 2.01µg/g to 1.81µg/g, while in case of MNH-886, the average protein level remained almost the same, which was 2.13µg/g to 2.12µg/g. These observations indicating that Bt toxin accumulation level was decreasing at 120 days in both varieties, but MNH-886 was a more stable Bt cotton variety concerning cry toxin expression and accumulation even under high-temperature environments.

Toxin interactions with temperatures, growth stages and variety

All parameters showed significant interactions with each other with reference to accumulation of Cry1Ac toxic protein in bollgard™ cotton. Figure-3 showed that both temperature and growth stages affects significantly for the accumulation of cry protein. In addition, varietal differences were also observed with reference to targeted protein. Results showed that protein level was increased upto a specific limit and started to decline with increase in temperature and advancements of growth stage. Maximum toxin level was observed at 35°C when plant reached at the age 100 days. Under low temperature environment, both cotton varieties perform equally. Similarly, toxin accumulation level was minimum in both varieties at lower temperature conditions i.e. 25°C when the plant's age was 60 days. These observations revealed that very hot and cold environmental conditions are negatively interacted with cry protein accumulation in leaf tissues of cotton. Likewise, primary and lateral growth stages also showed similar interactions with toxin level as temperatures. It was also observed, that MNH-886 showed higher toxic protein accumulation under all environmental conditions especially at 35°C and 40°C, and this toxin level was also stable even at lateral growth periods of cotton.

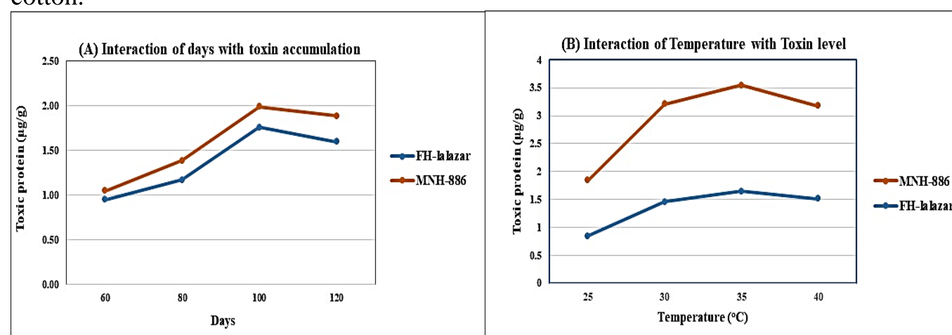

Figure 3. Interaction of different growth stages and temperature with the average accumulation of toxic protein in bollgard™ cotton leaves.

Discussion

Insects are one of the major problem in cotton production and improvement. These are not only lower the crop yield but its fiber quality also affected very badly. To cope with this challenge, Monsanto Company genetically modify cotton crop by the introduction of a bacterial *Cry1Ac* gene to create resistance against insect and is called as Bollgard™ cotton. This gene is responsible to develop crystalline (cry) protein which is toxic for chewing and sucking insects. Mon531 event of this cotton was formally approved for commercial cultivation by the Govt. of Pakistan in year 2010. Toxin level is very crucial factor for effective control of insects and it has been reported that it should not be less

than 1.9µg/g on a fresh weight basis (Karanthi et al., 2005). The present Bt cotton hybrids are descended from most popular GM cotton event i.e. Mon531 (Iqbal et al., 2013). Before quantify the toxin level in targeted plant, identification of responsible gene for this protein and its transformation event is an important factor. PCR is the primary tool routinely used to verify genetic transformation events at molecular level. Hence, Mon531 event successfully confirmed by isolating genomic DNA from the leaves and used in conventional PCR test using event specific primers. Amplification of 346bp indicated that both MNH-886 and FH-lalazar varieties have similar transformation event of *Cry1Ac* gene. Presence of transgene in plants does not always verify that it will be translated into specific protein. There are many external and internal plant mechanisms that can affect the expression and functionality of transgenes. Different molecular biology tools are used to confirm the transgene expression, but ELISA is the most reliable protocol to detect the target protein (Rochester, 2006). Hence, both qualitative and quantitative ELISA tests were performed to identify and quantify the cry protein in leaves respectively. The qualitative test is fast, cheap and easy to perform as it can give data only about the presence or absence of specific protein. So, Cry1Ac protein specific strips were used and results showed that both cotton varieties were positive for toxic cry protein (Figure 1B). Crop growth and yield is influenced by several internal and external factors which can also play a significant role in transgene expression. Experimental evidence for the effect of abiotic factors on cry protein expression is meager compared to influence of biotic factors. Of these, effect of soil, nutrients, heat, carbon dioxide and various other stresses are found to be important (Chen et al., 2005). In addition to heat stress, plant's age also plays a vital role in gene expression and protein accumulation. Level of toxic cry protein is changed with the plant growth and development (Kranthi et al., 2005; Dong & Li, 2007; Iqbal et al., 2013). Hence, it was decided to expose the plants to different heat stresses and to estimate the toxin level in top third leaf after every 20 days interval on each heat stress. Significant difference in cry toxin level was detected at 60, 80, 100 and 120 days under all temperature treatments. Maximum protein accumulation was found at 100 days at 35°C, while it was started to decrease at 120 days (Figure 2). Restricted growth with minimum protein accumulation was observed in plants placed at 25°C. This low protein level may be linked with slow cell division at low temperature. Performance of both Bt cotton varieties was almost similar at this temperature. Overall, MNH-886 accumulated higher level of cry protein in comparison with FH-lalazar and it was stable even at high temperatures (Figure-3). High and stable cry protein accumulation might be due to a reason that this variety is developed in southern Punjab having hot climate, hence it can more efficiently tolerate heat stresses. Declining pattern of cry1Ac toxic protein in different plant parts of cotton under variable temperatures and growth stages was also reported by Chen et al., 2000; Seki et al., 2001; Karanthi et al., 2005; Khan et al., 2017.

Conclusion

Effects of temperature stresses and different growth stages was investigated on Cry1Ac toxic protein accumulation in leaves of two bollgard^{TM} cotton varieties. Mon531 event of Bt cotton was verified using PCR technology while qualitative and quantitative ELISA was performed to identify and quantify the toxic protein respectively. Maximum toxin was found at 35°C when plant's age was 100 days. This level started to decline with rise

in temperature and advancements in growth stage. Overall, MNH-886 accumulated higher toxic protein at all temperature treatments and growth stages. This variety may be utilized as a heat tolerant source for the development of high temperature resistant Bt cotton genotypes.

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