Effect of Drought and Salinity Stress on the Expression Level of *Cry1Ac* Endotoxins in Transgenic Bt Cotton

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Cotton is a vital fiber crop and major agricultural product worldwide. It was genetically engineered with Cry genes from *Bacillus thuringiensis* (Bt), producing insecticidal proteins called Cry (Bt) toxins. Bt cotton efficacy depends on the expression level of these toxins, which abiotic stress negatively affects. This study examined how salinity and drought stress influence Cry1Ac endotoxin levels in transgenic Bt cotton. Initially, Cry1Ac transgene presence was confirmed using polymerase chain reaction (PCR) and immunostrip assays. Genotypes were then subjected to varying levels of salinity and drought stress under controlled conditions. Bt toxin levels were quantified using a commercial ELISA kit. Results showed that increasing drought and salt stress led to a decline in Bt protein expression. Toxin concentrations in genotypes MNH-886 and Bt-121 varied across different exposure durations (days three and six) during salinity treatment. FH-113 and 3701 genotypes exhibited variable Bt protein expression in response to drought. Specifically, genotype FH-113 exhibited higher toxin levels under drought conditions (20% PEG) compared to genotype 3701. These findings indicate that genetic background influenced Bt toxin expression under drought stress. In conclusion, increasing salinity and drought result in decreasing Cry1Ac toxin levels, which may negatively impact insect resistance efficacy of Bt cotton.

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INTRODUCTION

Cotton is a major fibre crop worldwide with numerous industrial and economic applications. Pakistan ranks fourth globally in cotton production, and cotton accounts for approximately 55% of the country's export earnings (Rana *et al.* 2020; Ashraf *et al.* 2024). Because of its substantial contribution to the gross domestic product (GDP), it is regarded as a key pillar of Pakistan's economy and a major fibre crop globally (Hussain 2002; Rana *et al*. 2020). Cotton production is affected by several challenges, among which insect pest infestation is a major constraint, accounting for approximately a 13% reduction in global cotton productivity (Chen *et al.* 2003). The *Lepidopterans*, which include *Spodoptera*, *Pectinophora gossypiella*, *Earias insulana*, and *Helicoverpa*, are among the most destructive pests affecting cotton production (Sulá *et al.* 2025). Genetically engineered Bt cotton was developed by introducing the Bt gene from *Bacillus thuringiensis* into cotton plants to protect crops against pests (Chen *et al.* 2003).

Insect control in Bt cotton is directly associated with the level of insecticidal endotoxins synthesized by the engineered *Cry* genes (Gutierrez and Ponsard 2006). A reduction in toxin levels results in ineffective insect control, and the targeted insect population may develop cross-resistance to Cry toxins (Tabashnik *et al.* 2008). Abiotic environmental conditions, such as drought, salinity, temperature, waterlogging, and nutrient deficiency, significantly influence on the expression level of Bt proteins in genetically modified cotton (Benedict *et al.* 2006; Luo *et al*. 2008; Luo *et al*. 2019; Huang *et al.* 2024). Salinity stress results in nutritional imbalance (Munns and Tester 2008), a negative osmotic effect on metabolism, and the toxicity of Na^+ and Cl^- ions, which collectively reduces plant growth and yield (Sairam and Tyagi 2004). Salt stress has been reported to significantly reduce Bt protein accumulation in the expanded leaves of Bt plants (Wang *et al.* 2018). Salinity stress has been shown to reduce total protein content and Bt protein levels by up to 7.2% and 11 to 22%, respectively, within a 7 to 12-day growth period (Gehlot *et al.* 2005). Salinity decreased the protein content of Bt by 50 to 72% when combined with waterlogging stress (Huang *et al.* 2024).

Similarly, drought is a major environmental stress that reduces plant growth and productivity (Buragohain *et al*. 2024). In response to drought stress, plants release reactive oxygen species (ROS), which limit enzyme activity, uncoil DNA strands, degrade lipids or proteins, and bleach pigments (Foyer and Noctor 2005). It is well known that drought stress and nitrogen deficiency have considerable effects on the level of *Cry1Ac* toxin expression (Traore *et al.* 2000; Bruns and Abel 2003). Furthermore, the transgenic Bt cotton genotype (*Gossypium hirsutum* L.), which is susceptible to both salinity and drought stress, is severely damaged by these two environmental factors (Tabashnik *et al.* 2008). Other studies have shown a decrease in the expression of *Cry1Ac* toxin when genetically modified Bt cotton plants were exposed to water deficiency or high temperature (Gutierrez and Ponsard 2006). During the growing season of Bt cotton plants, a reduction in the expression level of Bt toxin due to various abiotic stimuli may result in insufficient control of targeted insect pests and, as a result, the development of cross-resistance in targeted insect pests to genetically modified Bt cotton (Liu *et al.* 2019).

In Pakistan, the impact of abiotic stresses on the expression level of the Cry1Ac gene in cotton has not been explored. Different degrees of salinity are present in approximately 40% of arable land (approximately 6.67 million ha) in Pakistan (Quereshi and Sarwar 2009), and salt accumulates at a rate of approximately 40,000 ha per year (Ashraf *et al.* 2024). The country has also experienced prolonged drought over the past

three years, which may have had an impact on the amount of the Bt gene expressed in cotton.

In this study, an experiment was conducted on transgenic Bt cotton to evaluate the effects of drought and salinity stress on the expression of the Bt protein. Drought stress was simulated using polyethylene glycol (PEG-6000), a non-penetrating osmotic agent that reduces the water potential of the growth medium without being readily absorbed by plant tissues, thereby mimicking water deficit conditions under controlled environments. The use of PEG to impose osmotic stress and simulate drought conditions is well established, as it allows precise regulation of water potential and induces physiological responses comparable to those observed under natural drought stress (Lawlor 1970; Michel and Kaufmann 1973).

Salinity stress was imposed using sodium chloride (NaCl), which is widely employed to simulate salt stress by increasing osmotic pressure and causing ionic toxicity and nutrient imbalance in plant cells (Munns and Tester 2008). The results of the present study provide a clear picture of the expression levels of Cry1Ac toxins under salinity and drought stress and will help to manage the applicability of insect pest control in local Bt cotton genotypes under these stress conditions through the adoption of proper agronomic practices.

EXPERIMENTAL

Materials

The research project was conducted at the National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Centre (NARC), Islamabad, Pakistan.

Plant materials

Transgenic *Bt* cotton varieties of local origin, *i.e.*, MNH-886, Bt-121, 3701, and FH-113, were used in the present study. The seeds were obtained from the National Institute of Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad, Pakistan.

Germination of transgenic BT cotton seeds

The seeds of all cotton varieties were sown in white polyethylene nursery polybags (approximately 5 × 8 cm), each filled with about 300 to 400 g of prepared soil, providing suitable conditions for seed germination and early seedling development. Each polybag contained five seeds and was maintained in a glasshouse at 35 °C ± 5 °C. All the experiments were conducted in triplicate. Leaf samples were collected from the plants after germination for the detection of the Cry1Ac gene through PCR and confirmation of the Cry1Ac toxin through an ImmunoStrip assay (Agdia Inc., Elkhart, IN, USA).

Methods

Detection of the Cry1Ac gene through PCR

The CTAB method was used to extract genomic DNA from fresh leaf tissues of each plant sample. Young leaves were ground in liquid nitrogen to obtain a fine powder, which was transferred to tubes containing preheated CTAB extraction buffer and incubated at 65 °C to lyse the cells and release DNA. Chloroform isoamyl alcohol (24:1) was added

and the mixture centrifuged to separate the phases. The aqueous phase containing DNA was transferred to a new tube, and DNA was precipitated with cold isopropanol. The DNA pellet was washed with 70% ethanol, air dried, and dissolved in TE buffer for further analysis (Sambrook and Russell 2001).

For the DNA conformation of the Cry1Ac gene, event-specific primers (Mon 531) containing both forward and reverse sequences were synthesized (Yang *et al.* 2005).
Mon531 F (Forward sequence): 5'AAGAGAAACCCCAATCATAAAA3'
Mon531R (Reverse sequence): 5'GAGAATGCGGTAAAGATACGTC3'

The PCR was performed using a Veriti® thermal cycler (Applied Biosystems, Foster City, California, USA). The cycle began with denaturation at 94 °C for 10 min, followed by annealing at 50 °C for 1 min and extension at 72 °C for 1 min. Finally, the final extension was set at 72 °C for 10 min. The amplified product was separated *via* a 1% agarose gel (Pelican Life Sciences, San Diego, CA, USA), and the gel was observed under UV light. Using a 1 kb ladder, the expected size of 346 kb was confirmed.

Confirmation of Cry1Ac toxin through the immunostrip assay

Immunostrip analysis was used to establish the presence of Cry1Ac and Cry2Ab toxins. Fresh leaf tissues (100 mg) were taken from transgenic plants, and samples were prepared according to the manufacturer's protocol (Agdia Inc., Elkhart, Indiana, USA) Briefly, the leaf tissue was homogenized in the extraction buffer provided in the kit, and the extract evaluated for the presence of Cry1Ac and Cry2Ab toxins. The ends of the strips labelled with "SAMPLE" were inserted into the plant extract present in the microtube. Care was taken during the reaction time to prevent the strips from entering the extract from above 0.5 cm (1/4 inch). When the control lines emerged after 3 min, the reaction was considered valid. The immunostrips employed in this study were specific to the Cry1Ac and Cry2Ab proteins. The reaction findings were reported as positive (+) if the test line emerged within the given time; otherwise, they were reported as negative (-).

Hoagland Solution Formulation and Seedling Transfer

The Hoagland solution is composed of macro- and micronutrients. To prepare this solution, macro- and micronutrients were added in the appropriate quantities. Tables 2.1 and 2.2 provide information on the composition of the Hoagland solution.Only plantlets confirmed positive for the Cry1Ac gene were selected for the experiment. These plantlets were carefully transferred from the soil to a hydroponic system containing Hoagland nutrient solution (composed of macro- and micronutrients as detailed in Tables 2.1 and 2.2).

Induction of Abiotic Stress

The experiment followed a factorial design to evaluate the expression of the Cry1Ac gene under two types of abiotic stress across distinct intervals.

Drought Treatment

Following genomic and proteomic validation of the Cry1Ac transgene *via* PCR and immunostrip assays, plantlets of the 3701 and FH-113 varieties were transferred to a hydroponic system containing Hoagland solution (Hoagland and Arnon 1950). Drought stress was subsequently induced by amending the nutrient media with calculated concentrations of polyethylene glycol (PEG) to establish four treatment levels: control (0%), 10%, 15%, and 20% PEG (Michel and Kaufmann 1973; Lawlor 1970). These

osmotic stress treatments were initiated three days post-transfer and re-applied after a six-day interval to ensure consistent stress levels across both genotypes. To determine the impact of drought on transgene performance, the expression levels of the Cry1Ac toxin were quantified independently for each genotype using sandwich Enzyme-Linked Immunosorbent Assay (ELISA) at two distinct intervals, 3 and 6 days post-treatment and compared against non-stressed control plants.

Salt Treatment

Following genomic and proteomic validation of the Cry1Ac transgene via PCR and immunostrip assays, plantlets of the MNH-886 and Bt-121 strains were transferred to a hydroponic system containing Hoagland solution. Salinity stress was subsequently induced by amending the nutrient media with calculated concentrations of NaCl to establish four treatment levels: control (0 mM), 100 mM, 150 mM, and 200 mM. These salinity stress treatments were initiated three days post-transfer and re-applied after a six-day interval to ensure consistent stress levels across both genotypes.

To evaluate the impact of salinity on transgene performance, the expression levels of the Cry1Ac toxin were quantified independently for each genotype using sandwich Enzyme-Linked Immunosorbent Assay (ELISA) at distinct intervals and compared against non-stressed control plants.

Quantification of Cry1Ac Toxins through Sandwich ELISA Sampling Stages

After stress treatment, samples were collected from the plantlets at 3- and 6-day intervals. For all of the samples, Bt endotoxin was quantified *via* sandwich ELISA.

Plant tissues

For analysis, germinated true leaf tissues from each plant were used.

Bt toxin quantification

To begin the quantification procedure, 20 mg of fresh leaf tissue was manually ground with the extraction buffer supplied with the manufacturer's kit (Envirologix Inc., Portland, Maine, USA).

Sandwich- ELISA Method

The plant samples (20 mg) were crushed at room temperature with 500 µL of extraction buffer, and the homogenised samples were then centrifuged for 3 to 4 min at 10,000 to 12,000 rpm. A 100 mL sample of the supernatant was collected and gently mixed with 800 mL of dilution buffer. For the measurement of Cry1Ac toxin in each sample, four calibrators (standards) were used: 25 ppb, 10 ppb, 1.5 ppb, and a control (provided by the manufacturer). To determine the unknown Bt toxin expression, each well received a standard volume of 100 µL. The remaining wells were filled with 100 µL of diluted samples *via* a pipette, as instructed by the manufacturer. The ELISA plate was shaken after 30 s, after which 100 µL of the enzyme conjugate (provided by the manufacturer) was added to each well.

The shaking process was repeated for 30 s and the plate was covered and incubated at room temperature for 1 h. Following incubation, the contents were removed from the wells, and the wells were washed with washing buffer. The wells were dried by inverting the plate onto dry tissue paper. Each well was then supplied with 100 µL of substrate. After incubation at room temperature for 30 min, a blue colour developed in the wells, which

changed from blue to orange after the addition of 100 µL of stop solution. Finally, the optical density of each sample was measured using a microplate reader (BIO RADiMark™, Hercules, California, USA) at a wavelength of 415 nm to calculate the Cry1Ac toxin level in each sample.

Statistical Analysis

The mean of three replicates, together with the standard deviation, was used to present the data. Tukey's test and one-way analysis of variance (ANOVA) were employed to compare treatment means. Graph Pad Prism Version 8.01 was used for analysis.

Table 1. Macronutrients Added to Hoagland Solution

Compounds	Stock Solution (M)	Stock Solution (g/L)	Stock Solution Volume (mL/L Final)	Element	Final Concentration of Element (µM)
KNO_3	1.00	101.10	6.0	N	16000
$Ca(NO_3)_2.4H_2O$	1.00	236.16	4.0	K	6000
$NH_4H_2PO_4$	1.00	115.08	2.0	C	4000
$Mg.SO_4.7H_20$	1.00	246.49	1.0	P	2000
_	_	_	_	S	1000
_	_	_	_	Mg	1000

Table 2. Micronutrients Added to Hoagland Solution

Compounds	Stock Solution (M)	Stock Solution (g/L)	Stock Solution Volume (mL/L Final)	Element	Final Concentration of Element (µM)
KCL*	50	3.728	1.0	Cl	50
H_3BO_3*	25	1.546	1.0	B	25
$MnSO_4.H_2O$*	2	0.338	1.0	Mn	2
$ZnSO_4.7H_2O$*	2	0.575	1.0	Zn	2
$CuSO_4.5H_2O$*	0.5	0.125	1.0	Cu	0.5
H_2MoO_4(85%MoO_3) *	0.5	0.081	1.0	Mo	0.5
Fe-EDTA**	20	6.922	1.0	Fe	20

*Prepare a combined stock solution of all; ** Must be prepared separately

RESULTS

Immunostrip Analysis for BT Toxin Detection

Four regionally modified Bt cotton genotypes were tested using ImmunoStrip assays for the detection of two economically important Cry genes, namely Cry1Ac and Cry2Ab. The results of the immunostrip analysis revealed that all transgenic Bt cotton genotypes were positive for the Cry1Ac endotoxin, whereas the Cry2Ab endotoxin (Bollgard-II event) showed negative reactions in all genotypes (Table 3 and Fig. 1).

Table 3. Immunostrip Analysis of Four Local Bt Cotton Genotypes

Serial No.	Genotype	*Cry1Ac*	*Cry2Ab*
1	*FH-113*	+	-
2	*MNH-886*	+	-
3	*BT .121*	+	-
4	*3701*	+	-

+ = *Bt* toxin presence, - = *Bt* toxin absence

Fig. 1. Immunostrip strips showing positive results for Cry1Ac in Bt cotton genotypes

PCR Analysis

The PCR analysis was performed to determine the presence of the Bt gene at the DNA level. The PCR data revealed that Mon531 was present in all tested Bt cotton genotypes. For the Mon531-positive genotypes, a 346-bp PCR product was obtained (Fig. 2).

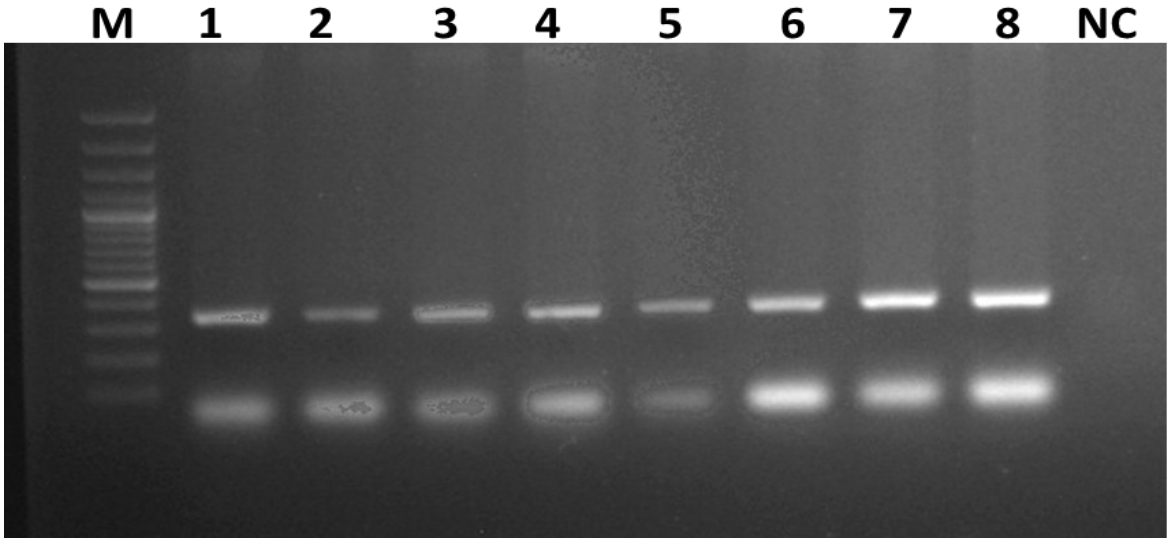

Fig. 2. Confirmation of the Bt gene in cotton (346 bp) through PCR. Lane M represents Marker/ladder, lane 1-2 showed MNH-886, Lane 3-4 showed Bt-121, lane 5-6 showed 3701, lane 7-8 showed FH-113 while lane NC= Negative controln cotton (346 bp)

ELISA Method

Compared with the control plants, all genetically modified Bt cotton genotypes that presented positive results in the Immunostrip for Bt toxins and for the Bt gene (Cry1Ac) gene in the PCR analysis were further analysed *via* sandwich ELISA to investigate Cry1Ac endotoxin expression levels under salt and drought stress treatments. The transgenic Bt cotton plants were subjected to salt and drought stress treatments to evaluate the expression patterns of the Cry1Ac gene in response to stress conditions.

Drought Stress Treatment

The germinated plants were subsequently transferred to Hoagland's solution for drought stress induction. Fresh leaf samples were collected from individual plants of the FH-113 and 3701 genotypes under control conditions and under 10%, 15%, and 20% PEG treatments after 3 and 6 days of exposure. The Cry1Ac toxin content in each sample was quantified *via* ELISA. After three days of drought stress, no statistically significant differences in Bt endotoxin levels were detected between the control plants and the plants of either genotype treated with 10% or 15% PEG. However, a significant reduction was detected in both genotypes under 20% PEG treatment compared with the control (Fig. 3A). By day six, the Bt endotoxin concentration decreased in a dose-dependent manner in both genotypes, with statistically significant reductions observed at 15% and 20% PEG (Fig. 3B).

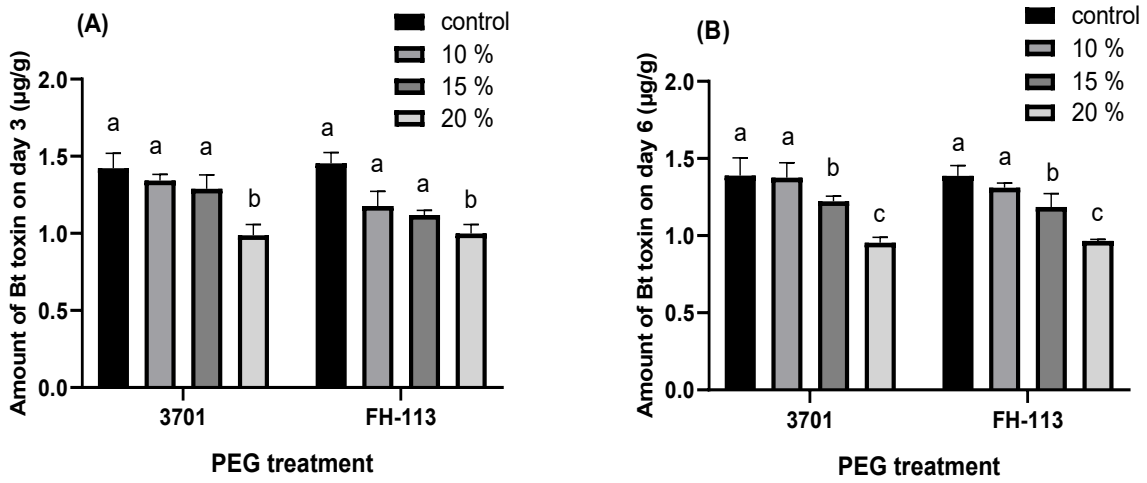

Fig. 3. Expression levels of Bt (Cry1Ac) endotoxin in the transgenic Bt cotton lines 3701 and FH-113 following exposure to drought stress induced by PEG at concentrations of 10%, 15%, and 20%: (A) a 3-day exposure period and (B) a 6-day exposure period. The data shown are the means of triplicate measurements, with the standard error of the mean (SEM) indicated. Statistically significant differences at $p < 0.05$ are represented by distinct alphabetical letters on the bars.

Salinity Stress Treatment

Salt stress assays were performed to assess the impact of salinity on Bt gene expression under control and stress conditions. Leaf tissues were harvested from individual plants in the control group and from plants subjected to 100 mM, 150 mM, and 200 mM NaCl stress after 3 and 6 days of treatment. After three days of exposure, no statistically significant difference in the Bt endotoxin content was detected between the control plants and plants of either genotype (Bt-121 or MNH-886) treated with 100 mM or 150 mM NaCl.

In contrast, a significant reduction in endotoxin levels was detected in both genotypes under 200 mM NaCl stress compared with those in the control group, as shown in Fig. 4A. After six days of salinity treatment, a dose-dependent decrease in the Bt endotoxin concentration was evident in both genotypes. The reductions were statistically significant at 100 mM, 150 mM, and 200 mM salt concentrations, as shown in Fig. 4B.

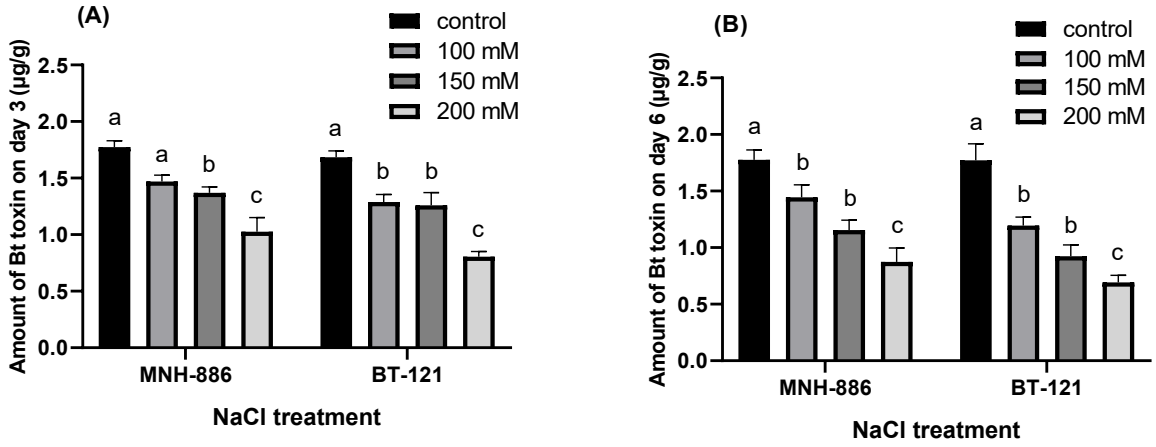

Fig. 4. Expression levels of Bt endotoxin (Cry1Ac) in the transgenic Bt cotton lines MNH-886 and BT-121 following exposure to salinity stress induced by NaCl at concentrations of 100 mM, 150 mM, and 200 mM: (A) a three-day exposure period and (B) a six-day exposure period. The data shown are the means of triplicate measurements, with the SEM indicated. Statistically significant differences at a $p < 0.05$ are represented by distinct alphabetical letters on the bars.

DISCUSSION

Drought, temperature, nutrient deficiency, waterlogging, and salinity are all abiotic stresses that have a substantial impact on the quality and agricultural production of many crops (Benedict *et al.* 2006; Luo *et al.* 2019; Huang *et al.* 2024). Endotoxin levels in various plant tissues play a dominant role in protecting plants against target pest attack in genetically modified Bt cotton (Likhitha *et al.* 2023). The concentration of Bt toxins in different areas of a plant does not remain constant over the course of its life cycle. Variations in endotoxin levels may impair the efficiency of Bt cotton against targeted insects (Luo *et al.* 2017). The effectiveness of Bt genes has been found to vary across cotton genotypes (Kranthi and Stone 2020), plant organs (Likhitha *et al.* 2023), transgene types, different environmental conditions (Tabashnik and Carrière 2019; Chen *et al.* 2024), and plant developmental stages (Likhitha *et al.* 2023).

This study focused on the effects of salt and drought stress on Bt cotton toxin levels and insect control efficiency. Salt stress has been shown to have a deleterious influence on the expression of different genes in rice plants (Pandit *et al.* 2011). Any observed change in gene expression could indicate a change in transgenic trait efficacy. Abrupt transgene inactivation results in the loss of a newly introduced characteristic (Rowan *et al*. 2025).

When exposed to drought stress, plants exhibit a range of responses at the whole-plant, cellular, and molecular levels (Ullah *et al*. 2017; Gupta *et al.* 2023; Wang *et al.* 2025). The degree and duration of drought stress, plant species, developmental stage of the plant (vegetative, germination, and reproductive phases), and organ and cell type under consideration all influence these responses. At the whole-plant level, the response of plants to drought stress is highly complex (Barkaoui and Volaire 2023). Drought stress reduces the concentration of Bt toxin because secondary elements involved in signal transduction, such as gibberellins, auxin, and ABA, are reduced, whereas stress-responsive molecules, such as heat shock proteins and ROS-protective proteins, are increased (Wang *et al.* 2025).

The results revealed that salt stress reduces the overall content of Bt toxins in the cotton genotypes tested. In this study, the Bt toxin level in leaves decreased approximately 44% under salt concentrations of 100, 150, and 200 mM, supporting previous findings that high salinity inhibits normal gene expression and thereby reduces Bt protein accumulation (Wang *et al.* 2018). High salt stress levels have a deleterious effect on hormone signals that respond to low water potential. High salt concentrations harm a variety of physiological functions (Balasubramaniam *et al.* 2023). A comparison of the control and salt-treated plants revealed that the concentration of endotoxin in the control plants was much greater than that in the salt-treated plants and decreased significantly at each salt stress level. Similarly, exposure to 200 mM NaCl resulted in approximately a 44 to 62% reduction in the Bt protein concentration with increasing salinity, as previously reported (Wang *et al.* 2018). The concentration of Bt toxins in the cotton genotypes MNH-886 and Bt-121 varied with both salt concentration and exposure duration (3 and 6 days). As the duration of salinity stress increased, the Bt protein concentration decreased.

The findings show that the Bt protein concentration remains high during the early stages of plant growth and that this high level of endotoxin is mostly due to the precipitation of a complex set of genes (Likhitha *et al.* 2023). Complex modifications in mRNA-binding proteins (Zhao *et al*. 2024) could partially explain the higher expression of the Bt gene (Cry1Ac). However, the present study on drought stress indicated that the expression level of Cry1Ac toxins was substantially lower than the USDA's recommendation for long-lasting insect resistance (1.5 µg/g fresh weight) in all of the tested lines. As a result of resistance development in targeted insects, such as Spotted, Pink, and American Bollworms, to the Bt toxin, the transitional and primarily low expression level of Bt compared with the USDA-recommended threshold poses a threat and prevents farmers from experiencing the long-term effectiveness of Bt technology. The results of the present study revealed that salt and drought stress are inversely related to Bt toxin levels, with increased salinity and drought stress resulting in a decrease in Bt protein levels. These reductions in Bt toxin levels could be attributed to the decomposition or synthesis of other proteins, as well as decreased nitrogen intake during salt stress (Guan *et al.* 2025). Plants produce diverse proteins in response to salinity that increase resistance to salt stress (Gehlot *et al.* 2005), whereas Bt protein, a soluble insecticidal protein, is produced primarily for insect control rather than improving tolerance to environmental challenges (Wang *et al.* 2018). In the present study, it was observed that the expression level of Cry1Ac toxin in leaf tissues gradually decreased with increasing drought stress. It was also concluded by (Wang *et al.* 2018) that the expression of Cry protein is considerably diminished by low soil moisture content and has the potential to adversely affect protein synthesis in Bt crops.

CONCLUSIONS

The applicability of Bt cotton is closely related to the expression level of Bt toxins, which varies with plant age and tissue type. Abiotic stresses, such as salinity and drought, can cause significant variation in Bt toxin expression, which not only reduces cotton productivity but also increases the risk of resistance development in targeted insect pests.

1. These effects are primarily associated with reduced Bt toxin expression in plant tissues. The results of the present study revealed that salinity and drought stress had detrimental effects on the expression level of Bt toxins in Bt cotton genotypes. Low levels of Bt toxin expression in Bt cotton may increase the risk of insect pest attack as a result of stress conditions.
2. The expression level of the Cry1Ac protein in local cotton genotypes under saline and drought stress was substantially lower than the USDA-recommended expression level (1.5 g/g fresh weight) for long-lasting insect resistance.
3. The levels of Bt toxin in both cotton genotypes declined significantly in response to the applied abiotic stress treatments in the experiment. To prevent pests from developing cross-resistance against Bt toxins, cotton genotypes exhibiting consistently low expression levels of Cry1Ac toxin are not recommended.
4. Cotton genotypes currently cultivated in Pakistan primarily express only the *Cry1Ac* gene. Genotypes with a package of Bt genes are recommended to prevent targeted pests from developing cross-resistance to Bt cotton and to enhance long-term effects.

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Competing Interests

The authors declare that there are no conflicts of interest.

Availability of Data and Material

All the data generated in this research work has been included in this manuscript.

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