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RESEARCH ARTICLE

Assessment of Cry1Ac and Cry2Ab expression endotoxins in transgenic cotton across growth periods and stages under semi-controlled conditions

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Abstract

Transgenic cotton has brought about a significant transformation in the management of bollworms. Nevertheless, a considerable challenge has emerged in the form of *Pectinophora gossypiella*for transgenic cotton. In current study, the detection and quantification of Cry1Ac and Cry2Ab protein in pot samples of different transgenic cotton cultivars encompassing single (Cry1Ac) and double gene (Cry2Ab) cultivars was conducted, employing bt strips and ELISA methods at three distinct time points, namely 40 days, 80 days, and 120 days after sowing, coinciding with different growth phases (i.e., leaves, squares, bolls, and seeds). The outcomes revealed that the highest toxin levels of Cry1Ac protein were present across all cultivars, whereas elevated levels of Cry2Ab protein were exclusively observed in MNH-1045 cultivar ranged from 0.05 ug/g to 1.46 ug/g. The findings highlighted visible differences in the concentrations of Cry1Ac and Cry2Ab proteins among all cultivars. The Cry1Ac concentrations ranged from 0.12 ug/g to 0.81 ug/g, 0.18 ug/g to 0.69 ug/g, 0.19 ug/g to 0.38 ug/g after 40 days, 80 days, and 120 days respectively, and ranged from 0.22 ug/g to 0.59 ug/g, 0.20 ug/g to 0.36 ug/g, 0.00 ug/g to 0.05 ug/g and 0.22 ug/g to 0.30 ug/g, in leaf, square, boll, and seed stage respectively. The concentration of Cry2Ab protein ranged from 0.04 to 1.46, 0.02 to 1.128, 0.01 to 0.28ug/g after 40 days, 80 days, and 120 days respectively, and ranged from 0.4 ug/g to 1.45 ug/g, 0.01 ug/g to 0.63 ug/g, 0.00 ug/g to 0.05 ug/g and 0.00 ug/g to 0.62 ug/g, in leaf, square, boll, and seed stage respectively. It was detected that the expression of toxins is depending upon the developmental stage of the crop and the duration of growth. This study's findings will aid entomologists and plant breeders in developing high-toxin cotton cultivars (especially expressing high at the boll stage) and strategies for global cotton production sustainability, such as refuge maintenance or hybrid development, to protect transgenic cotton from pink bollworm infestations.

Keywords: Bt cotton, Bt toxin, Cry1Ac detection, Cry2Ab detection, Growth periods, Growth stages, Quantification

Introduction

Globally, farmers allocate approximately 80% of the total pesticide usage towards the control of bollworms in cotton crops (Arshad & Suhail 2011). The extensive reliance on pesticides to combat cotton insect pests, particularly *Helicoverpa* armigera, has led to diminishing pesticide efficacy, reduced farmer income, escalated cotton production costs, and the emergence of resistance issues (Forrester et al., 1993; Yang et al., 2000; Lu et al., 2012). Researchers have identified an effective solution for mitigating insecticide resistance in these pests, which involves the utilization of biological insecticides

containing *Bacillus thuringiensis* (Schnepf et al., 1998). Subsequently, transgenic Bt cotton was developed, incorporating *Bacillus thuringiensis* endotoxins, specifically the Cry1Ac toxin, which has demonstrated remarkable effectiveness in managing cotton bollworms and other lepidopteran pests (Wilson et al., 1992; Flint et al., 1995; Hutchison 1999; Mendelsohn et al., 2003; Wu & Guo 2005). The commercialization of transgenic Bt cotton commenced in the USA in 1996 and spread to other nations, including China, Australia, Mexico, India, Argentina, South Africa, Colombia, and Brazil (James 2006). In Pakistan, it gained widespread adoption by 2010. Transgenic cotton offers multiple advantages to farmers, including reductions in pesticide usage, lower pest incidence, enhanced cotton sustainability, reduced production expenses, preservation of beneficial fauna, and increased yields (Godfray et al., 2010; Abedullah et al., 2015; Naseem and Qaim 2016). In China, the adoption of Bt cotton has resulted in a significant reduction in insecticide applications, ranging from 47% to 79% (Veettil et al., 2017).

*Pectinophora gossypiella*exhibits a heightened prevalence on transgenic cotton, primarily attributable to its monophagous feeding habits (Pogue 2004). The larvae of this pest primarily consume cotton flowers and bolls (Mapuranga et al. 2015). However, owing to the widespread cultivation of Bt cotton and the limited feeding on cotton by P. gossypiella, field populations of this species have developed resistance against transgenic cotton. Resistance evolution in pink bollworms has been documented in countries such as the USA, China, and India (Carrière et al. 2010; Tabashnik et al. 2013, 2019; Jin et al. 2015; Sansinenea 2019). In laboratory settings, researchers have identified four recessive mutant alleles of cadherin responsible for conferring resistance against Bt toxins, particularly Cry1Ac (Morin et al. 2003; Fabrick & Tabashnik 2012; Fabrick et al. 2014). Pakistan and other developing nations face an elevated risk due to the extensive cultivation of unapproved Bt cotton genotypes without adherence to proper refuge recommendations. In Pakistan, an outbreak of pink bollworm infestation on Bt cultivars was reported in Vehari (Abbas et al., 2016), although there have been no published reports of such infestations in Multan.

Several factors contribute to insect resistance against transgenic cotton, including the improper placement or implementation of refuge areas and the expression levels of Bt toxins. Initially, insect resistance to Bt cotton arose due to the inadequate availability of refuge plants (Huang et al., 2011). Furthermore, the potency and efficacy of Bt toxins are contingent upon the cultivars' capacity to produce Bt proteins during each growth stage and in various plant parts or tissues (Olsen et al., 2005; Wan et al., 2012; Zaman et al., 2015; Cheema et al., 2016; Khan et al., 2018). Notably, Bt toxin concentrations diminish with plant age or maturity (Fitt 1998; Holt 1998; Sachs et al., 1998; Greenplate et al., 2000; Adamczyk et al., 2001; Zaman et al., 2015). Research findings have consistently indicated that leaves exhibit higher levels of toxin expression compared to fruiting parts (Greenplate et al. 2000; Adamczyk et al., 2001; Abel et al., 2004; Bakhsh et al., 2010, 2012). This reduction in toxin expression correlates with the age of the transgenic crops (Brévault et al., 2012).

The management of insect resistance to Bt cotton can be effectively achieved through the cultivation of double-gene Bt cotton (incorporating Cry1Ac and Cry2Ab) or triple-gene Bt cotton (containing Cry1Ac, Cry2Ab, and Vip3Aa). This strategy is recognized as a vital approach for delaying the development of resistance to Bt (Kranthi et al., 2005). Some research studies have reported that the evolution of resistance can be significantly delayed through the use of refuges (Carrière and Tabashnik 2001; Tabashnik et al., 2003; Yang et al., 2014). The current investigation aims to assess the influence of various growth periods (specifically, 40 days, 80 days, and 120 days after sowing) and different plant parts (including leaves, square bolls, and seed cotton) on the expression of Cry1Ac and Cry2A protein within various single, double, and triple gene cotton cultivars. In contemporary agricultural scenarios, the outcomes of such research endeavors hold significant importance. These findings are expected to contribute valuable insights for the development of more effective strategies aimed at managing pink bollworm infestations during the cropping season.

Materials and Methods

Cotton cultivars

Various transgenic cotton cultivars were sourced from distinguished cotton research institutions, which encompassed the Cotton Research Station (CRS), Central Cotton Research Institute (CCRI), and Pakistan Central Cotton Committee (PCCC). These cotton cultivars comprised both single and double gene Bt cotton cultivars, as detailed in tab. 1. Subsequently, these selected cultivars were cultivated in pots under shade (semi-controlled conditions) within the premises of the University at MNS University of Agriculture Multan. From this initial selection, six cultivars were chosen to proceed with further trials, distinguished by their notably high Cry1Ac protein concentrations in leaf samples.

Table 1. List of transgenic Bt cotton cultivars grown in pots.

	Cultivar[1]	Source[3]
Cry1Ac	NS-211	PCCC
	CIM-598	CCRI
Cry1Ac + Cry2Ab	Weal-AG-201	PCCC
	Badar-3	PCCC
	CEMB Klean Cotton-6	PCCC
	MNH-1045	CRS

Note: 1. Cotton cultivars planted in pots include NS-211 (Cry1Ac Bt cotton), CIM-598 (Cry1Ac Bt cotton), Weal AG-201 (Cry1Ac+Cry2Ab Bt cotton), Badar-3 (Cry1Ac+Cry2Ab Bt cotton), CEMB Klean Cotton-6 (Cry1Ac+Cry2Ab Bt cotton), MNH-1045 (Cry1Ac+Cry2Ab Bt cotton).
2. C_5 and C_6 were transgenic "triple gene" cotton produces Cry1Ac and Cry2Ab Bt proteins as well as 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is responsible for glyphosate herbicide tolerance.
3. PCCC stands for Pakistan Central Cotton Committee, CRS stands for Cotton Research Station; CCRI stands for Central Cotton Research Institute.

Cry1Ac and Cry2Ab test by Bt Strips

Bt testing was conducted utilizing double-gene (Envirologix USA Quickstix Combo Strips for Cry1Ac & Cry2Ab) and triple-gene Bt strips (Envirologix USA Quickstix Combo Strips for Cry1Ac, Cry2Ab & CP4 EPSPS), following the prescribed procedure accompanying the strips to confirm the presence of Cry1Ac, Cry2Ab, and GTG genes. Leaf samples were collected from the 3rd node (Olsen et al. 2005; Yuan et al. 2012) at intervals of 40 days, 80 days, and 120 days from potted plants. Similarly, samples of leaves, squares, bolls, and seeds from potted plants were collected at various stages of plant growth. Following collection, all samples (including leaves, squares, bolls, and seeds) were promptly stored in a freezer set at -80°C.

For Bt testing, samples were carefully taken and manually ground in an extraction buffer composed of distilled water and the buffer supplied with the Bt strips, mixed in a ratio of 9:1. Subsequently, the ground samples were placed in Eppendorf tubes and subjected to Bt strip testing. After 30 seconds, the appearance or absence of a band on the strip indicated the presence or absence of Bt toxins, respectively.

Quantification of Cry1Ac and Cry2Ab by ELISA Kit

The collected samples, including leaves, squares, bolls, and seeds, were collected, leaf samples were collected from the 3rd node (Olsen et al., 2005; Yuan et al., 2012) at 40 days, 80 days, and 120 Days After Sowing (DAS). After collection, the samples were promptly kept at -80°C. To quantify the Cry1Ac and Cry2Ab proteins, Envirologix QuantiPlate ELISA kits for Cry1Ac and Cry2Ab (Envirologix, Portland, ME, USA) were used, by following the procedure described by Dohare and Tank, (2014). All necessary materials were supplied within the ELISA kit, except the wash and extraction buffers. The extraction and wash buffers were prepared by the guidelines provided in the ELISA kit instructions, specifically from the Envirologix Cry1Ac kit and Cry2Ab kit. The ELISA kits were employed following the methodology outlined by Dohare and Tank (Dohare and Tank 2014; Hanif et al., 2024).

Samples were taken and ground using a mortar and pestle, with the resulting mixture placed into Eppendorf tubes. To measure the results, an ELISA reader was utilized to obtain readings at 450 nm wave length. Non-linear regression was used to find polynomial equations from each standard curve for Cry1Ac and Cry2Ab. The Cry toxin concentration for each test sample was determined by solving each corresponding quadratic equation after the control/blank absorbance was corrected. The standards included with each ELISA kit were used to determine standard curves for each toxin, including 0 ng/g, 4 ng/g, 8 ng/g, and 16 ng/g, also denoted as parts per billion ppb, for Cry1Ac and 0 ng/g, 1 ng/g, 5 ng/g, and 10 ng/g (or ppb) for Cry2Ab.

Statistical analysis

All the data obtained from the ELISA kit were subjected to analysis by computing their respective means. Subsequently, means were compared employing Tukey's honestly significant difference test (LSD) test, facilitated by the use of Statistics 8.1 Software.

Results

Cry1Ac and Cry2Ab detection by Bt Strips

During the immunoblot strip test conducted on leaf samples, it was observed that all cultivar leaf samples consistently yielded false positive results (which means Cry1Ac protein was detected in samples) for the Cry1Ac protein in potted plants, across various time points after sowing (specifically, at 40 days, 80 days, and 120 days). However, false negative results were obtained for Cry2Ab protein (which means Cry2Ab protein was not detected in samples), except in the case of the MNH-1045 cultivar, as detailed in tab. 2.

Table 2. Immunoblot strip test of cultivars after different days of sowing.

	Cry1Ac			Cry2Ab			GTG[2]		
	40 DAS	80 DAS	120 DAS	40 DAS	80 DAS	120 DAS	40 DAS	80 DAS	120 DAS
NS-211[1]	3	+	+	NT[4]	NT	NT	NT	NT	NT
CIM-598	+	+	+	NT	NT	NT	NT	NT	NT
Weal AG-201	+	+	+	-	-	-	NT	NT	NT
(Badar-3	+	+	+	-	-	-	NT	NT	NT
CKC-6	+	+	+	-	-	-	+	+	+
MNH-1045	+	+	+	+	+	+	+	+	1

NOTE: 1. Cotton cultivars planted in pots include NS-211 (Cry1Ac Bt cotton), CIM-598 (Cry1Ac Bt cotton), Weal AG-201 (Cry1Ac+Cry2Ab Bt cotton), Badar-3 (Cry1Ac+Cry2Ab Bt cotton), CEMB Klean Cotton-6 (Cry1Ac+Cry2Ab Bt cotton), MNH-1045 (Cry1Ac+Cry2Ab Bt cotton).
2. GTG stands for glyphosate tolerance gene.
3. +ve or -ve stands for presence or absence of respected toxin.
4. NT stands for not tested because the cultivar does not contain it.

The analysis of Cry1Ac and Cry2Ab detection at various growth stages, including leaf, square, bolls, and seed cotton, consistently revealed false positive results for Cry1Ac protein across all cultivars, as presented in tab. 3. All tested cultivars show positive results for Cry1Ac at leaf, square, boll and seed stages. While for Cry2Ab, CKC-6 and MNH-1045 show positive results at leaf, and boll stages than other cultivars. However, at square stage, only MNH10-45 has more positive results than other treatments. As the crop advanced to the boll stage, cultivars displayed mixed responses, with some registering positive results for Cry2Ab and others negative results among tested cultivars. Finally, at the seed stage, all cultivars consistently demonstrated a positive response. Notably, all tested cultivar displayed positive results for GTG protein at leaf, square, boll, and seed stages.

Table 3. Immunoblot strip test of cultivars at different crop growth stages.

	Cry1Ac				Cry2Ab				GTG[2]			
	Leaf	Square	Boll	Seed	Leaf	Square	Boll	Seed	Leaf	Square	Boll	Seed
NS-211[1]	+[3]	+	+	+	NT[4]	NT	NT	NT	NT	NT	NT	NT
CIM-598	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT
Weal AG-201	+	+	+	+	-	-	+	+	NT	NT	NT	NT
(Badar-3	+	+	+	+	-	-	-	+	NT	NT	NT	NT
CKC-6	+	+	+	+	+	-	+	+	+	+	+	+
MNH-1045	+	+	+	+	+	+	+	+	+	+	+	1

NOTE: 1. Cotton cultivars planted in pots include NS-211 (Cry1Ac Bt cotton), CIM-598 (Cry1Ac Bt cotton), Weal AG-201 (Cry1Ac+Cry2Ab Bt cotton), Badar-3 (Cry1Ac+Cry2Ab Bt cotton), CEMB Klean Cotton-6 (Cry1Ac+Cry2Ab Bt cotton), MNH-1045 (Cry1Ac+Cry2Ab Bt cotton).
2. GTG stands for glyphosate tolerance gene.
3. +ve or -ve stands for presence or absence of respected toxin.
4. NT stands for not tested because the cultivar does not contain it.

Quantification of Cry1Ac and Cry2Ab by ELISA kit

Quantification of Cry1Ac and Cry2Ab protein after different days of sowing: Cry1Ac quantification was conducted on leaf samples obtained from both field-grown and potted plants of all cultivars at various time points following sowing/planting, specifically at 40 days, 80 days, and 120 days, as indicated in fig. 1. A highly significant difference was

detected in the levels of Cry1Ac protein among leaf samples of all cultivars at 40 days after sowing (F=56.4; df=5, 12; p<0.000) and 80 days after sowing (F=22.4; df=5, 12; p<0.000) under field conditions. However, no significant difference was observed at 120 days after sowing (F=9.05; df=5, 12; p<0.000). At the 40-day, leaf samples of CKC-6 cultivar exhibited the highest concentration of Cry1Ac protein compared to all other cultivars. The concentration of Cry1Ac protein (measured in micrograms per gram, ug/g) ranged from 0.12 ug/g to 0.81 ug/g, 0.18 ug/g to 0.69 ug/g, and 0.19 ug/g to 0.38 ug/g after 40 days, 80 days, and 120 days respectively. In some cultivars, concentration was decreased with respect to increasing days after sowing as NS-211, CIM-598, CKC-6, Badar-3, and MNH-1045. Conversely, Weal Ag-201 demonstrated elevated levels of Cry1Ac protein after 80 days and 120 days. However, Badar 3 demonstrated elevated levels of Cry1Ac protein after 80 and then decreased after 120 days.

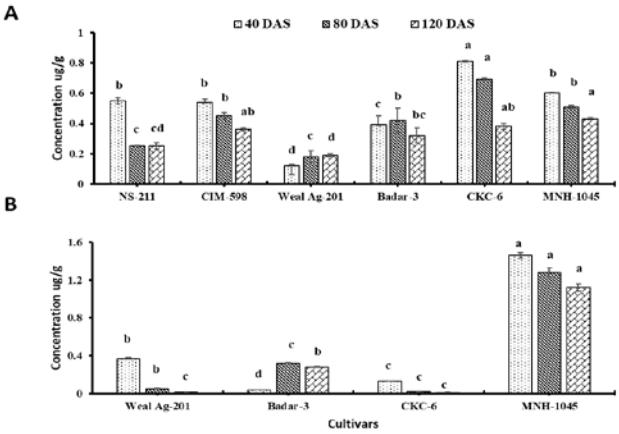

Figure 1. Mean quantification of Cry1Ac and Cry2Ab protein level (ug/g) of cultivars after different crop growth periods. A) Cry1Ac concentration in cotton leaves collected from test plots at 40, 80, and 120 days after sowing (DAS). B. Cry2A concentration in cotton tissues collected from test plots at 40, 80, and 120 DAS. Tested cultivars were NS-211 (Cry1Ac Bt cotton), CIM-598 (Cry1Ac Bt cotton), Weal AG-201 (Cry1Ac+Cry2Ab Bt cotton), Badar-3 (Cry1Ac+Cry2Ab Bt cotton), CEMB Klean Cotton-6 (Cry1Ac+Cry2Ab Bt cotton), MNH-1045 (Cry1Ac+Cry2Ab Bt cotton). DAS stands for days after planting. Entries in the same column, for quantification of Cry1Ac, followed by different letters are significantly different (P < 0.05) and the same letter shows not significantly different (P > 0.05). Means were separated using LSD test. Data shown are means of three replications; values are means ± standard errors.

The quantification of Cry2Ab protein levels in all cultivars was conducted at various time points following sowing/planting, specifically at 40 days, 80 days, and 120 days. The samples were collected from potted plants and are presented. Notably, an extremely significant difference was observed in the Cry2Ab protein levels within leaf samples of all cultivars at 40 days after sowing (F=1500; df=3, 8; p<0.000), 80 days after sowing (F=497; df=3, 8; p<0.000) and at 120 days after sowing (F=448; df=3, 8; p<0.000) for field-grown plants. Among all the cultivars, the MNH-1045 cultivar exhibited the highest protein levels of Cry2Ab than other cultivars after 40 days, 80 days, and 120 days. Furthermore, the concentration of Cry2Ab decreased with the passage of time in potted leaf samples of Weal Ag-201, CKC-6, and MNH-1045. However, Badar 3 demonstrated elevated levels of Cry1Ac protein after 80 days and then decreased after 120 days. The concentration of Cry2Ab protein (measured in micrograms per gram, ug/g) was ranging from 0.04 ug/g to 1.46 ug/g, 0.02 ug/g to 1.128 ug/g, and 0.01 ug/g to 0.28 ug/g after 40 days, 80 days, and 120 days respectively.

Quantification of Cry1Ac and Cry2Ab proteins at different growth stages: The quantification of Cry1Ac toxin levels was conducted across all cultivars at various growth stages, including leaf, square, boll, and seed, as outlined in fig. 2. A highly significant difference was observed in the levels of Cry1Ac protein among all cultivars at all stages, namely leaf (F=133; df=5, 12; p<0.000), square (F=140; df=5, 12; p<0.000), boll (F=M; df=5, 12; p=M), and seed (F=83.40; df=5, 12; p<0.000) under potted conditions. At the leaf stage, MNH-1045 displayed high levels of protein, while at the square stage, Badar-3 exhibited highest protein levels. Notably, at the bolls stage, lowest expression of Cry1Ac was observed among all tested cultivars. In the case of the seed stage, NS-211 demonstrated higher protein expression. Different concentrations of Cry1Ac protein were observed in samples obtained from potted plants, with levels ranging from 0.22 ug/g to 0.59 ug/g, 0.20 ug/g to 0.36 ug/g, 0.00 ug/g to 0.05 ug/g, and 0.22 ug/g to 0.30 ug/g, in leaf, square, boll, and seed stage respectively.

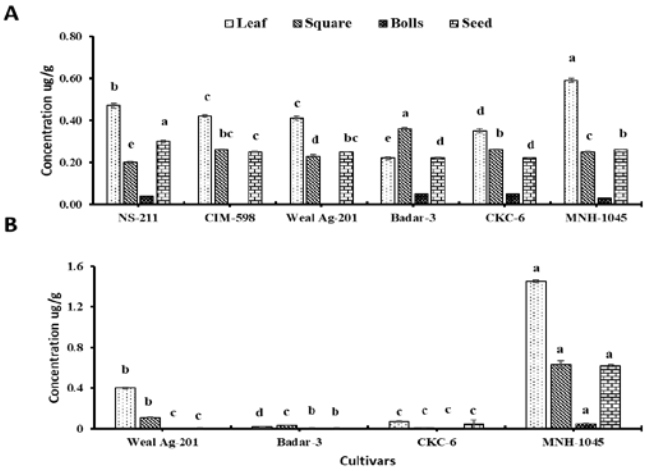

Figure 2. Mean quantification of Cry1Ac and Cry2Ab protein level (ug/g) of cultivars after different crop growth stages. A) Cry1Ac concentration in cotton tissues i.e. leaves, squares, bolls, and seeds collected from test plots at different growth stages. B. Cry2Ab concentration in cotton tissues i.e. leaves, squares, bolls, and seeds collected from test plots at different growth stages. Tested cultivars were NS-211 (Cry1Ac Bt cotton), CIM-598 (Cry1Ac Bt cotton), Weal AG-201 (Cry1Ac+Cry2Ab Bt cotton), Badar-3 (Cry1Ac+Cry2Ab Bt cotton), CEMB Klean Cotton-6 (Cry1Ac+Cry2Ab Bt cotton), MNH-1045 (Cry1Ac+Cry2Ab Bt cotton). Entries in the same column, for quantification of Cry1Ac, followed by different letters are significantly different (P < 0.05) and the same letter shows not significantly different (P > 0.05). Means were separated using LSD test. Data shown are means of three replications; values are means ± standard errors.

The quantification of Cry2Ab protein levels was determined across different cultivars at various growth stages, including leaf, square, boll, and seed. A highly significant difference was observed in the levels of Cry2Ab protein among all cultivars at all stages under field conditions, namely leaf (F=15959.8; df=3, 6; p<0.000), square (F=9665.77; df=3, 6; p<0.000), bolls (F=57.26; df=3, 6; p<0.000), and seed (F=37.68; df=3, 6; p=0.000). Among samples, only Weal Ag -201 and MNH-1045 exhibited the expression of Cry2Ab. Notably, the lowest level of concentrations of Cry2Ab protein was observed in other samples of tested cultivars, except MNH-1045 cultivar, with levels ranging from 0.05 ug/g to 1.45 ug/g. The MNH-1045 cultivar displayed high levels of Cry2Ab protein at the leaf stage, followed by the square, seed, and bolls stages. Conversely, in Weal Ag-201, the production of Cry2Ab protein decreased in plant growth stages from leaf to seed. The concentrations of Cry2Ab protein were ranging from 0.4 ug/g to 1.45 ug/g, 0.01 ug/g to 0.63 ug/g, 0.00 ug/g to 0.05 ug/g, and 0.00 ug/g to 0.62 ug/g, in leaf, square, boll, and seed stages respectively.

Discussion

Bollworm management initially relied on the cultivation of transgenic cotton without the use of chemical pesticides. However, due to the widespread cultivation of Bt cotton without the implementation of proper refuge strategies, bollworms developed resistance against Bt cotton (Gould 1998; Banerjee et al., 2017; Tabashnik & Carrière 2017). Another contributing factor to bollworm resistance is the variability in the expression of Cry1Ac (Jamil et al., 2021). The process of testing transgenic cotton involves cost-effective qualitative and quantitative techniques. In the current study, the qualitative detection of leaf samples from all cultivars consistently yielded positive results for the Cry1Ac protein, with the exception of Cry2Ab protein, which was detected only in the MNH10-45 and CKC-6 cultivar during various stages of crop growth (i.e., 40 days, 80 days, and 120 days). When testing different growth stages of all cultivars, the presence of Cry1Ac was observed in all samples of potted plants. However, the presence of Cry2Ab protein was only detected at the leaf, boll, and seed stages.

Quantitative analysis conducted through ELISA revealed variations in the concentration of the Cry1Ac protein among different cultivars during the crop growth period, specifically at 40 days, 80 days, and 120 days in potted plant leaf samples. Some cultivars exhibited higher Cry1Ac concentrations at 40 DAS, while others showed elevated concentrations at 80 DAS and 120 DAS. In contrast, the Cry2Ab protein concentration was highest at 40 DAS in the MNH-1045 cultivar but decreased as the crop growth period extended. Notably, in some cultivars, the concentration of the Cry2Ab protein was very low, even reaching zero. These findings align with previous research, which reported that the expression of the Cry1Ac protein in transgenic cotton decreases as the plant matures, impacting the efficacy of transgenic cotton against target insects later in the season (Zhang et al. 2001; Abel et al. 2004; Hanif et al., 2025). Indeed, there is evidence to suggest that the quantity of

insecticidal proteins in Bt cotton can vary depending on factors such as the plant's age, structure, and susceptibility to environmental challenges (Dong and Li 2007).

The quantitative analysis of Cry1Ac protein concentration revealed some noteworthy patterns among different cotton cultivars at various growth stages. Specifically, at the leaf and seed stages, samples of the MNH-1045 cultivar exhibited higher concentrations of Cry1Ac protein compared to other cultivars. The Badar-3 cultivar showed a higher concentration of Cry1Ac protein at the square stage than cultivars. While all cultivars had very low concentrations of Cry1Ac protein at the bolls stage. However, all cultivars have high concentrations at seed stage. Instead of Cry1Ac concentration, Cry2Ab concentration was highest in the MNH1045 cultivar at leaf, square, boll, and seed stages. Across different growth stages, Cry1Ac proteins were documented as being more abundant in leaves during the vegetative stage compared to the reproductive stage. These findings highlight the dynamic nature of protein expression in different cotton cultivars and at different stages of plant growth, which can have implications for pest management and the effectiveness of Bt cotton in controlling insect pests (Olsen et al., 2005). Another study demonstrated that the expression of the Cry1Ac toxin was higher in the leaves than at other stages, such as squares, flowers, and bolls (Tabashnik et al., 2003; Udikeri 2006; Likhitha et al., 2023). Drought and elevated temperatures significantly diminished the quantity of insecticidal protein in bolls (Zhang et al., 2021). The expression of the toxin decreased as the plant aged and as the crop seasons progressed in transgenic crops (Kranthi et al., 2005; Carrière et al., 2019). Hence, the decline in Bt toxin expression in Bt cotton with plant maturity resulted in increased survival of the target pests (Chen et al., 2000). Bt gene expression levels exhibit variability due to factors including the location of insertion, promoter sequences, environmental conditions, genetic bases, and genetic backgrounds (Guo et al., 2001; Adamczyk & Meredith 2004; Huang et al., 2011). Abiotic environmental conditions, such as soil salinity, elevated temperatures, waterlogging, nitrogen deficiency, and humidity, have an impact on the expression of the Bt gene (Chen et al., 2021; Rahman et al., 2022; Jehangir & Ali 2023).

Pink bollworm infestation in transgenic cotton results from decreasing toxin levels over time or the evolution of resistance in pink bollworms. Field-evolved resistance to transgenic cotton with Cry1Ac was reported in pink bollworms in India (Dhurua & Gujar 2011; Fabrick et al., 2014). Likewise, reports of pink bollworm infestations in double-gene Bt cotton (containing Cry1Ac and Cry1Ab) have emerged from India (Kranthi 2015; Mohan et al., 2016; Naik et al., 2018, 2020, 2021; Annepu et al., 2023). In Pakistan, pink bollworm infestation to green boll, locule, and open bolls of single, double, and triple gene cultivars was found to be highest (Hanif et al., 2025). Resistance evaluations were conducted against various endotoxins in seven major insect pest species on a global scale (Grimi et al., 2015). Other research results indicate that infestation in fruiting bodies was more pronounced in the BollGard-I genotype than in the BollGard-II genotypes (Gore et al., 2001; Jackson et al., 2003; Bheemanna et al., 2008; Onkaramurthy et al., 2016). Refuge planting has been recognized as the most effective strategy to delay pink bollworm resistance to transgenic cotton in the United States (Tabashnik 1994; Liu and Tabashnik 1997; Tabashnik et al., 2004, 2013). This strategy was also found effective in Pakistan (Hanif et al., 2025). This approach reduces selection pressure on target insect pests like pink bollworms and extends the lifespan of Bt cotton. It is recommended to plant refuge crops (non-Bt cotton) on a large scale alongside Bt cotton to manage pink bollworm incidence (Zaman et al., 2015).

Resistance monitoring in pink bollworms began in Arizona in 1996 (Tabashnik et al., 2000). In Pakistan, while outbreaks of pink bollworm damage on Bt cotton have occurred, resistance has not been confirmed through published articles. This study has identified cultivars with insufficient toxin levels to effectively control the pest. To address this issue, it is essential to increase the concentration of Cry1Ac and Cry2A genes in transgenic cotton by following the refuge strategy and all IPM (Integrated pest management) approaches (Hanif et al., 2025; 2025). Furthermore, the development of appropriate policies and strategies, such as implementing non-Bt cotton as a refuge with matching growth stages from flowering to fruiting, is necessary for environmentally friendly management of pink bollworm resistance to transgenic cotton.

Conclusion

The study aimed to assess Cry1Ac and Cry2Ab protein levels in transgenic cotton cultivars at various plant growth stages and periods. Overall, MNH1045 has higher concentrations of Cry1Ac than Cy2Ab among tested cultivars. It also concluded that the concentration of Cry1Ac protein in the leaf remained almost consistent across all growth periods and

stages but exhibited a decrease during the square and boll stages in all tested cultivars. While Cry2A levels declined over the developmental period and stages. While MNH-1045 had the highest concentration of Cry2Ab. However, protein levels depended on cultivars, growth periods (40 days, 80 days, and 120 days), and growth stages (leaf, square, bolls, and seed). Further research is needed to enhance toxin concentrations in transgenic cotton, especially at boll stage for better pest management, especially against pink bollworms in Pakistan.

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Conflicts of interest

There is no conflict of interest among authors. This manuscript does not contain any studies involving human participants and/or animals. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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