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Fronds light green, usually 1–3 together, oblong or ovate or orbicular, 2–5 × 0.13–0.09 mm, asymmetrical; two distinct papulae on the dorsal surface; nodal (where the veins converge) papillae smaller than apical one. Roots one; root sheath winged, ca. 0.01 × 0.02 mm. Inflorescence on two lateral pouches. Male flowers two, ca. 0.1 mm in length; anthers divaricate, bilocular, dehisce by transverse slit. Female flower composed of gynoecium; ca. 0.2 mm long; ovary globose, hyaline; style one, terminal. Fruit utricle.

Distribution in India: throughout the country.

Note: Though isolecto is said to be in BM (The Natural History Museum, London) and ZT (Edgenossische Technische Holochschule Zurich, Switzerland), the concerned databases of these herbaria do not show these types. But these types are included based on Landolt (l.c.).

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Bt Cry toxin expression profile in selected Pakistani cotton genotypes

Pakistan is ranked fourth among the top five cotton-producing countries in the world. About 70–80% of the cultivated area is under *Bt* cotton in Pakistan[1]. Bollworm (*Helicoverpa armigera*) is the major pest of cotton[2]. It causes 31.73–36.45% yield losses[3,4] and these are reduced by heavy pesticide application. There has been a tremendous increase in the import and use of pesticides. Consequently, about 7.7 billion rupees is spent on pesticides every year[5]. Considering the total pesticide usage (94,265 metric tonnes in 2007–08), 70% is being used exclusively on cotton. In addition to being a pollutant, pesticides are also hazardous to the farmers and livestock[1]. The large amount of money being spent on these chemicals can be avoided by planting *Bt* cotton.

From biosafety point of view, *Bt* biopesticides are better than chemical pesticides. Because the *Bt* toxins are highly specialized and have no negative effect on the environment[6]. The effectiveness of *Bt* cotton depends on the expression of insecticidal genes[7] and does not remain constant throughout the growing season[8]. The performance of *Bt* genes for controlling target insect pests varies according to the cotton varieties[9], age of the plant[10], different parts of the plant[11], types of gene and also the insertion sites of the gene into the DNA of target plants[12–14].

In Pakistan, the shift to *Bt* cotton was slow due to non-existence of necessary infrastructure. Plant breeders developed *Bt* varieties using local genotypes through backcrossing with alien *Bt* cotton varieties having the *Cry1Ac* gene of non-patented event (MON531) in Pakistan. In 2010, approximately 600,000 farmers cultivated *Bt* transgenic cotton varieties[15]. A study estimated that 81% and 90% were confirmed *Bt* varieties having only the *Cry1Ac* gene, in Sindh and Punjab provinces respectively[1]. The introduction of Bollgard-II event is expected soon as negotiations between the Government of Pakistan and Monsanto, USA are in progress.

To reduce the risk of resistance development in target insect pests against *Bt* cotton, there is a need to understand the variations in efficiency of *Bt* genes and their mechanisms. For this, advanced bioassay techniques like ELISA have been used to measure the quantity variations in the *Bt* Cry (crystal) proteins[16].

The main objective of the present study was to quantify actual *Bt* toxin levels in cotton genotypes at the growth stages when they are attacked by bollworm. The trend was also studied in plant parts (i.e. leaves and squares)

to spatial expression pattern for appropriate protection measures.

The present study was carried out at the National Institute for Genomics and Advanced Biotechnology, Islamabad during 2011.

Seeds of 46 local cotton genotypes were obtained from the Punjab Seed Council, Pakistan. Three to four seeds of each variety were planted in pots of 30 cm diameter having 3–4 kg mulching grey vertisol (clay 53%, organic carbon 0.75%; pH 9.0) under glasshouse conditions (Figure 1). The temperature of the glasshouse was set at 35°C. All other agronomic practices (i.e. irrigation and fertilization) were kept uniform.

Terminal plant leaves and squares were taken from individual plants per pot and stored at –80°C. Twenty milligram of each sample was weighted by electronic balance and ground manually using extraction buffer provided by the manufacturer.

Samples were prepared and confirmed by ImmunoStrip test for the detection of Cry1Ac, Cry2Ab and Cry1F following the manufacturer's instructions (Agdia Inc., USA). ImmunoStrips specific for Cry1Ac, Cry2Ab and Cry1F (Cata. #: STX06800; Cata. #: STX010300) were used in the study.

The positive lines detected by ImmunoStrip analysis were further analysed by Sandwich ELISA to quantify the Cry toxins. Sample preparation, calibrators (0, 1.5, 10, 25 ppb) (Calibrators were used to get standard regression line.) and Sandwich ELISA were performed according to the manufacturer's instructions (Envirologix). The optical density was calculated three times and the mean was recorded for each variety by adjusting the wavelength at 450 nm using a Microplate Reader (BIO RAD iMark^{TM}). Simple regression analysis was carried out using Microsoft Excel software to calculate toxin levels (µg/g) in different plant tissues.

Forty-six local cotton genotypes were analysed using ImmunoStrip for the detection of three *Bt* genes, viz. *Cry1Ac*, *Cry2Ab* and *Cry1F*. From the results (Table 1) it is clear that 70% (32/46) of *Bt* cotton genotypes showed positive reactions for *Cry1Ac* gene (Figure 2). Whereas for *Cry2Ab* and *Cry1F*, all the genotypes showed negative reactions. Hence 30% (14/46) of the total genotypes did not show any *Cry* gene and these were confirmed as non-*Bt* genotypes of cotton by ImmunoStrip analysis.

This may be due to low levels of Cry toxin that would not be in the range of detection. Similar results were obtained by Ali *et al.*[1], who reported that among 42 local *Bt* cotton genotypes, 34 were confirmed as *Bt* genotypes by ImmunoStrip analysis. This mixing (i.e., *Bt* and non-*Bt* genotypes) adversely affects the potential of *Bt* genotypes and will pose a threat to the environment.

Figure 1. Local cotton genotypes in a glasshouse.

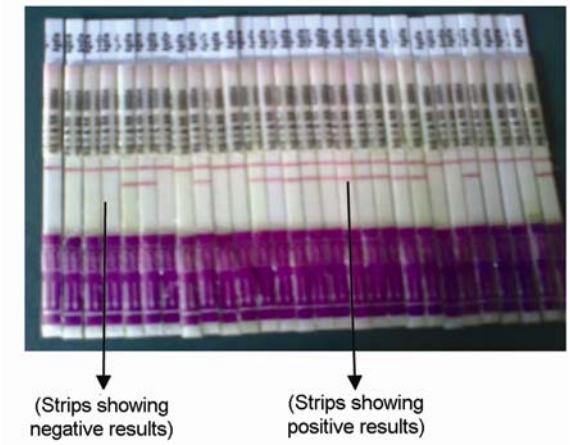

Figure 2. ImmunoStrip analysis for *Bt* toxin detection.

Table 1. ImmunoStrip analysis of 46 Pakistani cotton genotypes

Genotype	*Cry1Ac*	*Cry2Ab*	*Cry1F*
A1	+	–	–
A2	+	–	–
A3	+	–	–
A4	–	–	–
A5	–	–	–
A6	+	–	–
A7	+	–	–
A8	–	–	–
A9	–	–	–
A10	–	–	–
A11	+	–	–
A12	–	–	–
A13	+	–	–
A14	+	–	–
A15	+	–	–
A16	+	–	–
A17	+	–	–
A18	+	–	–
A19	+	–	–
A20	+	–	–
A21	–	–	–
A22	–	–	–
A23	+	–	–
A24	+		–
A25	–	–	–
A26	–	–	–
A27	–	–	–
A28	+	–	–
A29	–	–	–
A30	–	–	–
A31	+	–	–
A32	+	–	–
A33	+	–	–
A34	+	–	–
A35	+	–	–
A36	+	–	–
A37	–	–	–
A38	+	–	–
A39	+	–	–
A40	+	–	–
A41	+	–	–
A42	+	–	–
A43	+	–	–
A44	+	–	–
A45	+	–	–
A46	+	–	–

+, *Bt* genes present; –, *Bt* genes absent.

Table 2. Quantification of *Bt* toxins in leaf tissues at 85, 100 and 130 days after sowing (DAS)

Genotype	85 DAS			100 DAS			130 DAS		
	Cry1Ac (μg/g)	*Cry2Ab*	*Cry1F*	*Cry1Ac* (μg/g)	*Cry2Ab*	*Cry1F*	*Cry1Ac* (μg/g)	*Cry2Ab*	*Cry1F*
A1	0.963	–	–	0.911	–	–	0.875	–	–
A2	0.536	–	–	0.495	–	–	0.462	–	–
A3	0.902	–	–	0.846	–	–	0.812	–	–
A4	–	–	–	–	–	–	–	–	–
A5	–	–	–	–	–	–	–	–	–
A6	0.715	–	–	0.679	–	–	0.655	–	–
A7	0.259	–	–	0.199	–	–	0.176	–	–
A8	–	–	–	–	–	–	–	–	–
A9	–	–	–	–	–	–	–	–	–
A10	–	–	–	–	–	–	–	–	–
A11	0.856	–	–	0.805	–	–	0.774	–	–
A12	–	–	–	–	–	–	–	–	–
A13	0.529	–	–	0.489	–	–	0.459	–	–
A14	0.757	–	–	0.724	–	–	0.689	–	–
A15	0.519	–	–	0.494	–	–	0.475	–	–
A16	0.692	–	–	0.632	–	–	0.602	–	–
A17	0.604	–	–	0.571	–	–	0.549	–	–
A18	0.465	–	–	0.417	–	–	0.388	–	–
A19	0.409	–	–	0.367	–	–	0.337	–	–
A20	1.063	–	–	1.011	–	–	0.978	–	–
A21	–	–	–	–	–	–	–	–	–
A22	–	–	–	–	–	–	–	–	–
A23	0.981	–	–	0.941	–	–	0.902	–	–
A24	0.249	–	–	0.212	–	–	0.201	–	–
A25	–	–	–	–	–	–	–	–	–
A26	–	–	–	–	–	–	–	–	–
A27	–	–	–	–	–	–	–	–	–
A28	0.855	–	–	0.812	–	–	0.786	–	–
A29	–	–	–	–	–	–	–	–	–
A30	–	–	–	–	–	–	–	–	–
A31	0.526	–	–	0.497	–	–	0.468	–	–
A32	0.401	–	–	0.365	–	–	0.332	–	–
A33	0.701	–	–	0.654	–	–	0.634	–	–
A34	0.705	–	–	0.665	–	–	0.642	–	–
A35	0.586	–	–	0.511	–	–	0.497	–	–
A36	0.997	–	–	0.934	–	–	0.912	–	–
A37	–	–	–	–	–	–	–	–	–
A38	0.738	–	–	0.689	–	–	0.665	–	–
A39	0.563	–	–	0.512	–	–	0.478	–	–
A40	0.806	–	–	0.751	–	–	0.729	–	–
A41	0.905	–	–	0.863	–	–	0.833	–	–
A42	0.771	–	–	0.712	–	–	0.687	–	–
A43	0.928	–	–	0.875	–	–	0.851	–	–
A44	0.505	–	–	0.456	–	–	0.436	–	–
A45	0.538	–	–	0.487	–	–	0.466	–	–
A46	0.487	–	–	0.425	–	–	0.401	–	–

The Cry1Ac toxin expression was computed in Pakistani cotton genotypes with respect to age and tissues. Thirty-two transgenic *Bt* genotypes that expressed positive reactions for *Cry1Ac* gene by ImmunoStrip analysis were further subjected to Sandwich ELISA for quantification of Cry1Ac toxins. Leaves were taken from individual plants at 85 DAS (days after sowing), 100 DAS and 130 DAS. Varying levels of Cry1Ac toxin were observed using ELISA assay (Table 2; Figure 3).

From the results it is obvious that the Cry1Ac toxin level in leaf tissues ranged from 0.249 to 1.063 μg/g at 85 DAS; 0.212 to 1.011 μg/g at 100 DAS, and 0.201 to 0.978 μg/g at 130 DAS, on fresh weight basis. Two genotypes, A20 and A36, expressed the highest amount of Cry1Ac toxins, i.e. 1.063 and 0.997 μg/g respectively, at 85 DAS; 1.011 and 0.934 μg/g respectively, at 100 DAS and 0.978 and 0.912 μg/g respectively, at 130 DAS. The data revealed that the Cry1Ac toxin levels differed in all *Bt* cotton genotypes. These results are in agreement with those obtained by Kranthi *et al.*[17], who reported that Cry1Ac toxin levels differed significantly among the various genotypes and decreased with the passage of time. The appropriate plant parts containing sufficient levels of Cry proteins play significant roles against harmful insect-pests[18]. The *Bt* toxins in different tissues of the cotton plant vary throughout its life cycle. Due to this, the tolerance of cotton plants towards target pests (i.e. lepidopteran

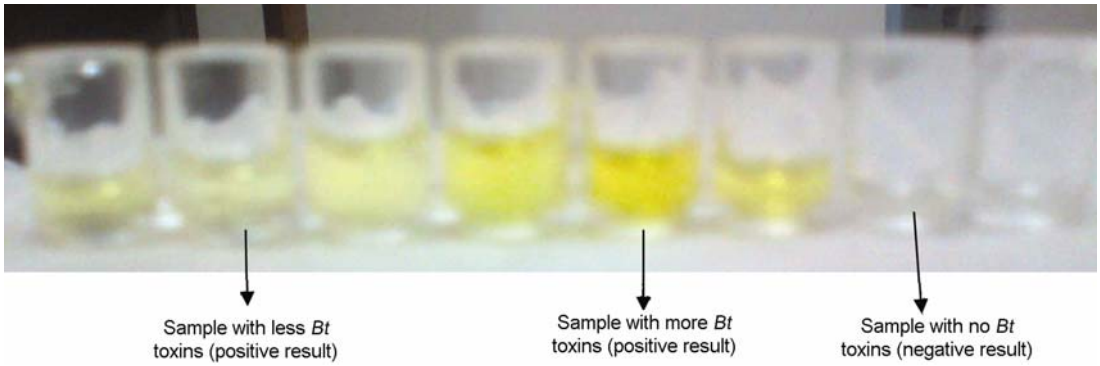

Figure 3. Quantification of *Bt* toxin by Sandwich ELISA.

Table 3. Quantification of *Bt* toxins in square tissues at 85, 100 and 130 days after sowing (DAS)

Genotype	85 DAS			100 DAS			130 DAS		
	Cry1Ac (μg/g)	*Cry2A*	*Cry1F*	*Cry1Ac* (μg/g)	*Cry2A*	*Cry1F*	*Cry1Ac* (μg/g)	*Cry2A*	*Cry1F*
A1	0.364	–	–	0.305	–	–	0.286	–	–
A2	0.407	–	–	0.371	–	–	0.352	–	–
A3	0.452	–	–	0.411	–	–	0.391	–	–
A4	–	–	–	–	–	–	–	–	–
A5	–	–	–	–	–	–	–	–	–
A6	0.366	–	–	0.322	–	–	0.301	–	–
A7	–	–	–	–	–	–	–	–	–
A8	–	–	–	–	–	–	–	–	–
A9	–	–	–	–	–	–	–	–	–
A10	–	–	–	–	–	–	–	–	–
A11	0.588	–	–	0.538	–	–	0.512	–	–
A12	–	–	–	–	–	–	–	–	–
A13	0.373	–	–	0.338	–	–	0.311	–	–
A14	–	–	–	–	–	–	–	–	–
A15	0.398	–	–	0.349	–	–	0.324	–	–
A16	0.461	–	–	0.417	–	–	0.385	–	–
A17	0.388	–	–	0.349	–	–	0.317	–	–
A18	0.482	–	–	0.439	–	–	0.418	–	–
A19	0.351	–	–	0.301	–	–	0.276	–	–
A20	0.433	–	–	0.398	–	–	0.372	–	–
A21	–	–	–	–	–	–	–	–	–
A22	–	–	–	–	–	–	–	–	–
A23	0.601	–	–	0.562	–	–	0.531	–	–
A24	0.287	–	–	0.228	–	–	0.209	–	–
A25	–	–	–	–	–	–	–	–	–
A26	–	–	–	–	–	–	–	–	–
A27	–	–	–	–	–	–	–	–	–
A28	0.288	–	–	0.264	–	–	0.232	–	–
A29	–	–	–	–	–	–	–	–	–
A30	–	–	–	–	–	–	–	–	–
A31	0.407	–	–	0.387	–	–	0.359	–	–
A32	0.462	–	–	0.424	–	–	0.401	–	–
A33	0.347	–	–	0.312	–	–	0.287	–	–
A34	0.436	–	–	0.397	–	–	0.369	–	–
A35	0.398	–	–	0.367	–	–	0.336	–	–
A36	0.341	–	–	0.308	–	–	0.278	–	–
A37	–	–	–	–	–	–	–	–	–
A38	0.456	–	–	0.412	–	–	0.387	–	–
A39	0.359	–	–	0.323	–	–	0.299	–	–
A40	0.391	–	–	0.365	–	–	0.338	–	–
A41	0.534	–	–	0.504	–	–	0.472	–	–
A42	0.358	–	–	0.314	–	–	0.276	–	–
A43	0.398	–	–	0.367	–	–	0.342	–	–
A44	–	–	–	–	–	–	–	–	–
A45	–	–	–	–	–	–	–	–	–
A46	0.438	–	–	0.412	–	–	0.387	–	–

pests) may decrease[17,19]. It is clear from the results that the leaves have higher levels of Cry1Ac protein than the squares[17], as reported in several earlier studies[17,20,21].

Squares were taken from individual plants (at 85 DAS, 100 DAS and 130 DAS) of 28 *Bt* cotton genotypes. The expression of Cry toxins was checked using ELISA assay. The results are shown in Table 3.

It is apparent from the results that all the transgenic *Bt* genotypes vary from each other with respect to Cry1Ac toxin levels that ranged from 0.287 to 0.601 µg/g at 85 DAS; 0.228 to 0.562 µg/g at 100 DAS and 0.209 to 0.531 µg/g at 130 DAS, on fresh weight basis. The two *Bt* genotypes, namely A11 and A23 showed the highest level of Cry1Ac toxin, i.e. 0.588 and 0.601 µg/g respectively, at 85 DAS; 0.538 and 0.562 µg/g respectively, at 100 DAS, 0.512 and 0.531 µg/g respectively, at 130 DAS. The appropriate level of Cry toxins at a specific time is crucial for the protection of plants against specific insect-pests. The levels of Cry1Ac protein were higher during the initial developmental stages of the plant[9] and decreased as the plant attained maturity[17]. Then it is clear that the Cry1Ac toxin levels at 85 DAS of cotton were higher than 100 DAS and 130 DAS. This means that the amount of Cry1Ac toxins decreased gradually as the plants attained maturity. Several factors are responsible for the varying levels of Cry1Ac toxin. Mainly, it may be due to variation in gene expression. Variation in the expression of *Bt* gene occurs due to its base sequences, copy number, the promoter used and gene incorporation point into the DNA of target *Bt* varieties[22,23]. The decrease in Cry1Ac proteins at late developmental stages was due to low expression of mRNA[19] and also due to variations in methylation status of the promoter (35S) region[24].

The potency of *Bt* genes in transformed *Bt* cotton genotypes fluctuates with age and different parts of the plant. This variation in expression of insecticidal genes has become hindrance for cotton growers to adopt *Bt* cotton because lower toxin levels will not only increase the cost of production but also cause the development of resistance in target insect-pests to GM cotton crop. This variability in efficiency is mostly due to the lower levels of Cry proteins in the plant tissues. The variation in Cry protein expression is a complicated process. It may be due to over-expression of the insecticidal genes at earlier stages of plant growth, that ultimately results in gene silencing at later stages. Research must be focused on developing new promoters that will help in the regular production of endotoxins throughout the growing season of the cotton plant. Moreover, transgenic *Bt* cotton genotypes must be developed with promoters that increase the expression of *Bt* genes, especially of cotton fruiting parts that are more vulnerable to target insect-pest attack.

The results show that quantitative levels of *Bt* toxin in all local genotypes were high at 85 DAS. It decreased at 100 DAS and also at 130 DAS. Hence it is clear that Cry toxin contents decrease when the plant attains maturity. Appropriate plant protection measures should be taken at late stage of the cotton-growing season. The low level of toxin expression in squares/bolls and in later stages of growth may make the crop susceptible to bollworm attack. This will inturn affect the economics of the farming community.

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