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STATUS OF *BT* COTTON CULTIVATION IN MAJOR GROWING AREAS OF PAKISTAN

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Abstract

A survey of 10 districts in Sindh and 11 in Punjab was conducted during cotton growing season of 2007-08. Samples were collected from a total of 126 locations. Two samples from each location were subjected to ImmunoStrip analysis for the detection of *Bt-Cry* protein which revealed that 81% (34/42) and 90% (76/84) samples from Sindh and Punjab provinces, respectively, were positive for *Bt* protein and harbored *CryIAc* gene. However, none of the sample was found to have *Cry2Ab* and *Cry1F* genes. The samples were further analyzed to confirm their transgenic nature by ELISA for *npt- II* (Kanamycin) selection marker gene encoded protein. Another limited survey was conducted in 2009-10 to re-assess the situation. Both surveys revealed that *Bt* transgenic cotton is widely grown in the cotton growing areas of Sindh and Punjab. This is the first science based study to estimate the extent of *Bt* cotton spread in the country

Introduction

Bacillus thuringiensis, commonly known as *Bt* is a bacterium that occurs naturally in soil. It has been used as a biological pesticide for more than 50 years (Qaim & Zilberman, 2003). First generation of transgenic cotton encompassed plants with single insecticidal *Bt* genes (Ferry *et al*., 2006). Several *Bt* transgenic crops including corn, tomato, canola, potato, chickpea, egg plant and cotton have been field tested in USA, Argentina, Canada, India and Australia. Cotton crop has been transformed with various forms of *Bt* gene producing crystal protein toxin. *Bt* genes (*Cry IAc*, *Cry IAb*, *Cry2Ab*, and *Cry 1F)* have commercialized in more than 18 cotton producing countries (Forrester, 2008). In 2005, farmer community celebrated 10 years anniversary of the release of first GM crop harboring *Bt* gene (Anon., 2005). The year 2009 represents the 14th planting season since *Bt* cotton was first commercially grown in 1996. China and India are the two major growing countries. More than 50% of the global cotton area is now under genetically modified cotton (James, 2008, 2009). In India, area under *Bt* cotton has increased to 8.4 million hectares in 2009 exceeding that of China's 3.4 million hectares (James, 2009).

The *Cry* protein in *Bt* cotton provides insecticidal activity against many Lepidopteran species. Planting of Bollgard-I cotton since 1996 in the US resulted a reduction in insecticide use of 2.7 million pounds of insecticidal active ingredients (Carpenter & Gianessi, 2001). US cotton growers planting Bollgard cotton showed a 260 million pound increase in cotton production per year which resulted in an estimated $99 million increase in net income in 1999 (Carpenter & Gianessi, 2001). There are a number of secondary benefits associated with the reduction in insecticide (non biological) use, which include enhanced populations of beneficial insect and wild life, reduced potential runoff of insecticides, and improved safety for farm workers by reducing potential exposure. Similarly in China, recently it has been reported that plantation of *Bt* crops is also beneficial in reducing pests of nearby non-*Bt* crops (www.aaas.org/news/releases/

2008/0918china_cotton.shtml). In short, *Bt*. toxins and their genes are a unique resource for agricultural system Pakistan is among the top 5 cotton producing countries in world. Among the total pesticide usage (94265 metric tone in 2007-08), predominantly (70%) is being used exclusively on cotton. In addition to pollutant, it is also hazardous for the farmers, lady pickers and livestock. Most farmers do not have protective clothes and other material while applying insecticides with small back-pack sprayers. Poisoning from such chemicals and even death was a big problem for farmers in China, India and Pakistan. There is potential advantage of using *Bt* cotton. However, commercial release of any GM crops in Pakistan including cotton requires clearance of EPA/NBC (Environment Protection Agency/National Biosafety Centre;http//;www.environment.gov.pk), IPO (Intellectual Property Right Organization;http;//www.ipo.gov.pk) and Ministry of Food and Agriculture. Development of infrastructure for the new bodies resulted in the delay of approval process of *Bt* cotton by various public/private sector (Zafar, 2007). The vacuum was filled by un-authorized/un-approved *Bt* cotton seed because of strong demand of farming community of the country. This resulted in coverage of large areas under cultivation of un-approved *Bt* cotton seed. Earlier some limited surveys have been conducted by NGOs in Pakistan by just asking questions to farming community while no technical study has been conducted so far. In this regard, extensive surveys were conducted in major cotton growing areas of Sindh and Punjab provinces (Fig. 1). The main objectives were to investigate the presence/absence of *Cry* toxin in *Bt* transformed cotton and to explore the class of *Cry* gene/toxin.

Materials and Methods

Survey was conducted in the cotton growing areas of Sindh and Punjab province (as shown in Fig. 1) in collaboration with Pakistan Central Cotton Research Institutes, Sakrand and Multan during July-August 2007-08 and June-July 2009. In 2007-08, ten districts of Sindh were surveyed and samples were collected for *Bt* transgenic cotton from 42 locations. Similarly eleven districts in the Punjab province were surveyed and samples were collected from 84 different locations. Six districts were surveyed in Sindh and Punjab in 2009-10. Five samples were taken randomly from each location. Of these 5 samples, 2 were further randomly selected for laboratory testing and the remaining three were stored as reference samples.

ImmunoStrip analysis test procedure: Sample preparation and test were carried out according to manufacture's instructions (Agdia Inc. USA). ImmunoStrips specific for *Cry1Ab/Ac-Cry2Ab* (Cata. #: STX06800) and *Cry1F* (Cata. #: STX010300) were used in the testing procedures.

Qualitative rating for expression intensity: For the rating of expression intensity of *Cry* genes in positive samples, time factor was taken into consideration. Lines appeared within 5, 15 and 30 minutes were scored in qualitative term as high (+++) medium (++) and low (+) toxin concentration. Lack of expression was denoted by negative sign (-).

Enzyme linked immuno sorbent analysis (ELISA) for *npt-II*: Locations showing negative reaction for samples in ImmunoStrip analysis (Tables 1 & 2) were further analyzed to detect plant transformation selectable marker enzyme neomycin phosphotransferase (*npt-II* protein) by ELISA.

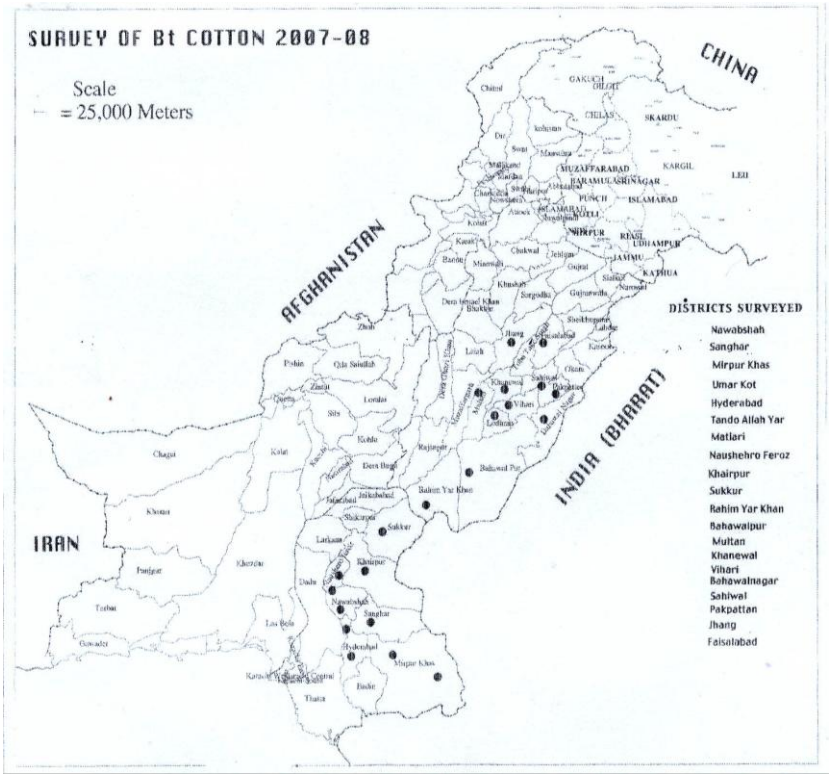

Fig. 1. Survey areas of Punjab and Sindh. Black dots showing the route of survey.

ELISA procedure: Preparation of samples, standards and the procedure was performed following manufacturer's instruction (Agdia Inc. USA). Wells were examined visually and scored as high (+++), medium (++), low (+) concentration and lack of expression (–). The reading (O.D.) was measured using ELISA plate reader (imark® Bio-Rad, USA) at 450 nm.

Results and Discussion

Survey 2007-08: It was observed during that 80% of cotton growing area in Sindh province had come under *Bt* cotton cultivation. Among the surveyed districts, Sanghar had the maximum area under *Bt* cotton (>90%). An exotic source of *Bt* cotton named as Australian *Bt* showing high susceptibility to CLCuV (60-100%) was found to be prevalent in the Sanghar district in 2007-08. Less number of pesticide sprays and protection from boll worms was reported by the farmers who experienced cultivation of *Bt* cotton in the previous year (2006-07). A proportion of 10-20% off type cotton plants was observed in these fields. However, this was not the case with Aus-*Bt* cotton. A highly uniform population was found in most of the fields planted with this genotype. The introduction of Aus-*Bt* cotton in Sindh province, which is highly susceptible to CLCuV, is posing a severe threat to cotton production in the country as it will increase the inoculum pressure. This may play a role in the evolution of new virus strain as it has happened in case of "Burewala virus", a new potent strain of cotton leaf curl virus resulting in huge losses to cotton crop in the country.

Table 1. ImmunoStrip analysis of samples collected from 42 locations of Sindh (2007-08).

S. No.	Genotype	Source	*Bt* genes expression intensity					
			Cry1Ab/Ac		*Cry2Ab*		*Cry1F*	
			1	**2**	**1**	**2**	**1**	**2**
1.	Aus-*Bt*	Nawabshah	+++	+++	-	-	-	-
2.	Aus-*Bt*	Nawabshah	+++	++	-	-	-	-
3.	Local- *Bt*	Nawabshah	-	-	-	-	-	-
4.	Local- *Bt*	Sanghar	++	++	-	-	-	-
5.	Local *Bt*	Sanghar	-	-	-	-	-	-
6.	*Bt* 109	Sanghar	++	++	-	-	-	-
7.	Local-*Bt*	Sanghar	++	++	-	-	-	-
8.	Local-*Bt*	Sanghar	++	++	-	-	-	-
9.	Aus-*Bt*	Sanghar	++	++	-	-	-	-
10.	Aus-*Bt*	Sanghar	++	-	-	-	-	-
11.	Local-*Bt*	Sanghar	++	++	-	-	-	-
12.	Local-*Bt*	Sanghar	++	++	-	-	-	-
13.	Aus-*Bt*	Mir Pur Khas	++	-	-	-	-	-
14.	Local-*Bt*	Mir Pur Khas	++	++	-	-	-	-
15.	Local-*Bt*	Mir Pur Khas	+++	-	-	-	-	-
16.	Local-*Bt*	Umerkot	+++	-	-	-	-	-
17.	Local-*Bt*	Umarkot	++	++	-	-	-	-
18.	Local-*Bt*	Umarkot	+++	+++	-	-	-	-
19.	Local-*Bt*	Mir Pur Khas	+	-	-	-	-	-
20.	Local-*Bt*	Mir Pur Khas	+++	+++	-	-	-	-
21.	Local-*Bt*	Hyderabad	-	-	-	-	-	-
22.	Local-*Bt*	Hyderabad	+++	+++	-	-	-	-
23.	Aus-*Bt*	Hyderabad	+++	++	-	-	-	-
24.	Aus-*Bt*	Hyderabad	++	++	-	-	-	-
25.	Local-*Bt*	Tando Allah Yar	-	-	-	-	-	-
26.	Local-*Bt*	Tando Allah Yar	-	+	-	-	-	-
27.	Local-*Bt*	Tando Allah Yar	++	++	-	-	-	-
28.	Local-*Bt*	Tando Allah Yar	+++	+++	-	-	-	-
29.	Local-*Bt*	Tando Allah Yar	-	-	-	-	-	-
30.	Local-*Bt*	Tando Allah Yar	+++	+++	-	-	-	-
31.	Local-*Bt*	Matiari	+++	-	-	-	-	-
32.	Local-*Bt*	Matiari	++	-	-	-	-	-
33.	Local-*Bt*	Matiari	+++	-	-	-	-	-
34.	Local-*Bt*	Khairpur	-	-	-	-	-	-
35.	Local-*Bt*	Khairpur	+++	+++	-	-	-	-
36.	Local-*Bt*	Khairpur	+	-	-	-	-	-
37.	Local-*Bt*	Khairpur	++	++	-	-	-	-
38.	Local-*Bt*	Sukkar	+++	+++	-	-	-	-
39.	Local-*Bt*	Sukkar	+++	+++	-	-	-	-
40.	Aus-*Bt*	Nowshero Feroze	+	-	-	-	-	-
41.	Aus-*Bt*	Nowshero Feroze	-	-	-	-	-	-
42.	Local-*Bt*	Nowsharo Feroze	-	-	-	-	-	-

+++ = Expression within 5 minutes, ++ = Expression within 15 minutes, + = Expression within 30 minutes, - Lack of expression

Table 2. ImmunoStrip analysis of samples collected from 84 locations of Punjab (2007-08).

S. No.	Genotype	Source	*Bt* genes expression intensity					
			Cry1Ab/Ac		*Cry2Ab*		*Cry1F*	
1.	*BT*-121	Khanewal	++	++	-	-	NT	NT
2.	BR-196	Khanewal	++	++	-	-	NT	NT
3.	IR-1524	Khanewal	++	++	-	-	NT	NT
4.	IR-901	Khanewal	+++	+++	-	-	NT	NT
5.	FH-113	Khanewal	+	-	-	-	NT	NT
6.	MG-3	Khanewal	+++	+++	-	-	NT	NT
7.	ASR-10	Khanewal	++	++	-	-	NT	NT
8.	ASR-5	Khanewal	++	+++	-	-	NT	NT
9.	ASR-2	Khanewal	+	+	-	-	NT	NT
10.	IR-448/8	Khanewal	+++	+++	-	-	NT	NT
11.	*BT*-Karishma	Khanewal	+++	+++	-	-	NT	NT
12.	IR-2456	Khanewal	+++	+++	-	-	NT	NT
13.	IR-2389	Khanewal	+++	+++	-	-	NT	NT
14.	IR-2379	Khanewal	+++	+++	-	-	NT	NT
15.	IR-2403	Khanewal	+++	+++	-	-	NT	NT
16.	BR-102	Khanewal	+++	+++	-	-	NT	NT
17.	BR-103	Khanewal	+++	+++	-	-	NT	NT
18.	IR-448/10	Khanewal	+++	+++	-	-	NT	NT
19.	MG-2	Khanewal	+++	+++	-	-	NT	NT
20.	448/133	Khanewal	++	++	-	-	NT	NT
21.	MG-I	Khanewal	+++	+++	-	-	NT	NT
22.	IR-2316	Khanewal	++	-	-	-	NT	NT
23.	2865	Khanewal	+++	++	-	-	NT	NT
24.	*Bt*-121	Khanewal	++	++	-	-	NT	NT
25.	*Bt*-121	Khanewal	++	+	-	-	NT	NT
26.	*Bt*	Lodhran	+++	+++	-	-	NT	NT
27.	IR-901	Lodhran	++	++	-	-	NT	NT
28.	*Bt*-121	Lodhran	+	+	-	-	NT	NT
29.	IR-2403	Lodhran	+++	++	-	-	NT	NT
30.	*Bt*-113 (FH-113)	Lodhran	++	++	-	-	NT	NT
31.	*Bt* -222	Lodhran	-	-	-	-	-	-
32.	*Bt* -216	Lodhran	+	+	-	-	NT	NT
33.	*Bt* -121	Lodhran	++	++	-	-	NT	NT
34.	IR-1524	Lodhran	-	-	-	-	-	-
35.	*Bt*-121	Bahawalpur	+++	+++	-	-	NT	NT
36.	*Bt*-196	Bahawalpur	++	-	-	-	NT	NT
37.	CP 140	Bahawalpur	+	-	-	-	NT	NT
38.	*Bt*-196	Bahawalpur	+++	++	-	-	NT	NT
39.	IR-1524	Bahawalpur	-	-	-	-	-	-
40.	*Bt*	Bahawalpur	++	++	-	-	NT	NT
41.	*Bt*-196	Rahim Yar Khan	++	++	-	-	NT	NT
42.	*Bt*-496	Rahim Yar Khan	++	-	-	-	NT	NT

Table 2. (Cont'd.).

S. No.	Genotype	Source	*Bt* genes expression intensity					
			Cry1Ab/Ac		*Cry2Ab*		*Cry1F*	
43.	*Bt*-473	Rahim Yar Khan	-	-	-	-	-	-
44.	*Bt*-446	Rahim Yar Khan	++	++	-	-	NT	NT
45.	*Bt*-102	Rahim Yar Khan	++	++	-	-	NT	NT
46.	IR-1524	Lodhran	++	+	-	-	NT	NT
47.	ASR-2	Lodhran	+++	+++	-	-	NT	NT
48.	*Bt*-473	Lodhran	+++	+++	-	-	NT	NT
49.	ASR-10	Lodhran	++	++	-	-	NT	NT
50.	IR-1000	Lodhran	++	++	-	-	NT	NT
51.	ASR-3	Lodhran	+++	+++	-	-	NT	NT
52.	IR-1573	Lodhran	+++	+++	-	-	NT	NT
53.	IR-901	Multan	+	-	-	-	NT	NT
54.	NIBGE-I	Multan	++	+	-	-	NT	NT
55.	ASR-6	Multan	++	++	-	-	NT	NT
56.	*Bt*-121	Multan	+++	++	-	-	NT	NT
57.	CP-1401	Multan	-	-	-	-	-	-
58.	ASR-10	Multan	+	+	-	-	NT	NT
59.	ASR-12	Multan	++	++	-	-	NT	NT
60.	*BT*-121	Vehari	++	++	-	-	NT	NT
61.	*Bt*-121	Vehari	++	++	-	-	NT	NT
62.	*Bt*	Vehari	++	++	-	-	NT	NT
63.	*BT*-121	Vehari	++	++	-	-	NT	NT
64.	*Bt*	Vehari	-	-	-	-	-	-
65.	*Bt*-493	Bahawalnagar	-	-	-	-	-	-
66.	*Bt*-121	Bahawalnagar	+++	+++	-	-	NT	NT
67.	*Bt*	Sahiwal	++	++	-	-	NT	NT
68.	*Bt*-501	Pakpatan	+	+	-	-	NT	NT
69.	*Bt*	Pakpatan	++	++	-	-	NT	NT
70.	*Bt*-121	Pakpatan	++	++	-	-	NT	NT
71.	*Bt*-473	Pakpatan	-	-	-	-	-	-
72.	*Bt*	Pakpatan	++	++	-	-	NT	NT
73.	*Bt*-121	Jhang	++	+	-	-	NT	NT
74.	*Bt*	Jhang	++	++	-	-	NT	NT
75.	*Bt*-448\8	Jhang	+++	++	-	-	NT	NT
76.	IR-1524	Jhang	+	+	-	-	NT	NT
77.	*Bt*	Jhang	++	++	-	-	NT	NT
78.	*Bt*-196	Jhang	+	+	-	-	NT	NT
79.	*Bt*	Jhang	++	++	-	-	NT	NT
80.	ASR-12	Jhang	+++	+++	-	-	NT	NT
81.	ASR-7	Jhang	+	+	-	-	NT	NT
82.	*Bt*-14	Jhang	+	-	-	-	NT	NT
83.	*Bt*-133	Jhang	+	+	-	-	NT	NT
84.	IR-901	Jhang	+++	+++	-	-	NT	NT

+++ = Expression within 5 minutes, ++ = Expression within 15 minutes, + = Expression within 30 minutes, - = Lack of expression, NT = Not tested

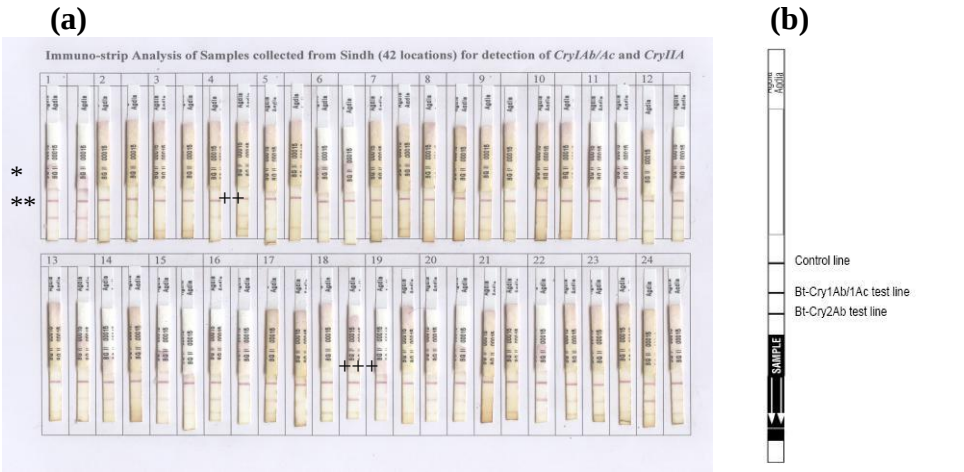

Fig. 2. (a) ImmunoStrip analysis: *control line, **test line for *CryIAc*, +++ high concentration, ++ medium concentration, + low concentration. (b)-General image of Strip STX006800.

Almost 50% cotton growing area has been observed to be occupied by *Bt* cotton in the Punjab province in 2007-08. Maximum area under *Bt* cotton was located in Khanewal, Vehari and Bahawalnagar districts (60-65%). Of all the genotypes planted in field, *Bt*-121 (Neelum 121) occupied major area (more than 40%), and was relatively better than other *Bt* genotypes as regard to uniformity. A wider range of segregation (20-30%) among other *Bt* cotton varieties was observed in some of the fields.

Laboratory analysis for the detection of *Cry* protein in Sindh: The ImmunoStrip analysis revealed that samples collected from 81% (34/42) locations were +ve for *Cry* protein (Fig. 2a, Table 1). All samples expressing positive reaction harbor *CryIAc* gene (Fig. 2a) whereas no reaction was detected for *Cry2Ab* and *Cry1F* genes (Figure not shown for *Cry1F*). Regarding the intensity of protein expression at 34 locations, samples from 14 locations (41%) showed high concentration (+++) of toxin, whereas samples from 15 locations (44%) exhibited medium (++) and remaining 5 locations (15%) showed very low (+) concentration (just visible) of toxin. Of the positive locations (34) for the presence of *Cry* protein, both of the tested samples (from each location) of 23 locations (68%) yielded positive response whereas 11 locations (32%) gave one negative and one positive reaction. This may be attributed to chances of seed mixing or monogenic segregation in back crossed breeding population. In Australian *Bt* cotton (local name), a high level of toxin protein expression was detected. However, seed mixing in this genotype has been practiced and samples collected from Nowshero Feroze exhibited negative result, whereas samples collected from districts Mirpur Khas, Umer kot and Sukkhar (10 different locations) proved to be positive. In the district Sanghar, where *Bt* cotton has been widely planted, samples collected from 8 locations, showed positive reaction and one location was negative (sample from local *Bt* cotton).

Fig. 3. ELISA for the detection of *npt-II* protein. Wells with blue color show positive reaction.

Laboratory analysis for the detection of *Cry* protein in Punjab: The ImmunoStrip analysis revealed that samples collected from 90% (76/84) locations were positive for *Cry* protein (Fig. 2a, Table 2). Samples expressing positive reaction harbor *CryIAc* gene whereas no reaction was detected for *Cry2Ab* and *Cry1F* genes (figure not shown for *Cry1F*). As regard to the intensity of protein expression at 76 positive locations, samples from 27 locations (36%) showed high (+++) concentration of toxin whereas 35 locations (46%) exhibited medium (++) and remaining 14 locations (18%) showed very low (+) concentration (just visible) of toxin. In fact, many factors are responsible for this variable expression such as gene position effect, reduced gene silencing resulting from homology with some endogens (co- suppression), number of integrated copies, genetic background of parent species, different 3' end regions (Matzke & Matzke, 1994; Sachs *et al.*, 1998) influence of plant's development and environment etc., (Down *et al*., 2001). At a time, one or a combination of these factors may be operating to drive the transgene expression. The low level of *Cry* gene expression may not be a good sign because it is not desirable for durable resistance and may result in the development of cross resistance in target insect pest.

Population uniformity in terms of toxin expression seems to be higher in the Punjab than Sindh province. Of the 84 locations, 76 displayed positive response for the presence of *Cry* protein (Table 2). Out of these, 69 locations (91%) yielded positive response for both samples whereas 7 locations (9%) gave negative reaction for one sample only. This may be attributed to chances of seed mixing or monogenic segregation. The toxin level in the widely spread genotype (*Bt*-121) was found to be satisfactory (+++, ++) confirming its action or activity against boll worms. Similar kind of observation was narrated by growers of region. A very strong and high level of expression (+++) was detected in the genotypes MG-1, MG-2 and MG-3, probably having the same origin and event. Genotypes started with "IR" and "ASR" prefix exhibited a reasonably high level of *Cry* toxin. Therefore, protection against the target insect pests can be expected from these genotypes. Samples collected from districts Khanewal and Jhang (37 different locations) turned out to be positive (Table 2). In district Lodhran, samples collected from 16 locations showed positive reaction with the exception of two locations, which were negative. From districts Multan, Vehari, Bahawalpur, Rahim Yar Khan and Pakpattan, sampling was done from 30 different locations. Samples only from one location in each of these districts showed negative reaction for *Cry* protein.

Table 3. Expression intensity of *npt-II* detected by ELISA (2007-08).

S. No.	Sample	Reaction	ELISA Reading
1.	Non *Bt*	-	0.083
2.	Non *Bt*	-	0.077
3.	Positive control	+	0.129
4.	Positive control	+	0.114
5.	Aus-*Bt* (Nawabshah)	+++	0.286
6.	Aus- *Bt* (Hyderabad)	+++	0.328
7.	Blank(Buffer)	-	0.096
8.	Blank(Buffer)	-	0.086
9.	Location#3 (Nawabshah)	-	0.102
10.	Location #5(Sanghar)	+++	0.249
11.	Location# 21 (Hyderabad)	+++	0.290
12.	Location# 25(Tando A. Yar)	-	0.087
13.	Location# 29(Tando A. Yar)	-	0.093
14.	Location# 34(Khairpur)	-	0.105
15.	Location# 41 (Nowshero Feroze)	+	0.170
16.	Location# 42 (Nowshero Feroze)	-	0.090
17.	Location# 31 (Lodhran)	+	0.131
18.	Location# 34 (Lodhran)	+++	0.267
19.	Location# 39 (Bahwalpur)	-	0.104
20.	Location# 43 (R.Y. Khan)	++	0.222
21.	Location# 57(Multan)	-	0.061
22.	Location# 64 (Vehari)		0.093
23.	Location# 65 (Bahawalnagar)	+	0.133
24.	Location# 71(Pakpattan)	-	0.065

+++ = Expression within 5 minutes, ++ = Expression within 15 minutes, + = Expression within 30 minutes, - = Lack of expression

ELISA for the detection of *npt-II* protein: In genetic engineering of cotton, *npt-II* gene harboring the trait of breakdown of antibiotic (kanamycin) is routinely employed as selectable marker. The detection of *npt-II* could be used to ascertain the transgenic nature of samples. Of the 8 locations in Sindh province, samples from Sanghar, Hyderabad and Tando Allah Yar proved to be of transgenic whereas others were negative (Fig. 3, Table 3). In Punjab province, samples from 4 locations, two from Lodhran and one each from Rahim Yar Khan and Bahawalnagar were transgenic whereas others were confirmed as negative. The negative response during ImmunoStrip analysis of these samples may be attributed to very low expression level of *Cry* protein that is not within the detection limit.

Survey 2009-10: A limited survey of *Bt* cotton was conducted in the cotton growing areas of Sindh and Punjab provinces during June, 2009-10. The surveyed districts were Shaheed Benazir Bhuttoabad (Nawabshah), Sanghar and Mirpur Khas in Sindh and Multan, Khanewal and Vehari in Punjab province. Almost 100% cotton area was observed to be occupied by *Bt* cotton genotypes in the regions of Sindh. The proportion of *Bt* cotton area was also significantly increased in Punjab in this year (80%) compared to the year 2007-08 (50%). Severe attack of CLCuV was observed in all cotton fields irrespective of *Bt* cotton. Almost same genotypes of *Bt* cotton were found as were in year 2007-08. It means farmers have maintained and multiplied the same seed materials for

two years (2007-2009). Results of immunoStrip analysis revealed the presence of *Cry1Ac* in all of the positive samples. 100% of samples collected from both provinces proved to be positive for *Cry1Ac* (Table 4). *Cry2Ab and Cry1F* could also be not detected in this year which shows the absence of Bollgard-II® and Wide Strike® in cotton growing regions of Pakistan. Variation for the presence of *Cry1A*c was obvious in few of samples. In conclusion, *Bt* transgenic cotton is widely grown in the cotton growing areas of Sindh and Punjab. The area expanded to almost 100% in Sindh and 80% in Punjab. The level of *Bt* gene expression varied from low to high indicating that source of seed is different. The detection of one *Cry* gene (*Cry1Ac*) provides an evidence about the presence of only Bollgard-I® and absence of Bollgard-II® (*CryIAc+Cry2Ab*) and Wide Strike® (*CryIF*) in Pakistan. A part of the samples within a location giving negative response indicates the possibility of seed mixing/segregation for *Bt* gene. This impurity may lead to serious concerns such as host resistance breakdown, low credibility of *Bt* transgenic cotton in the minds of agricultural farming community. Cultivation of almost 36 un-approved cotton varieties is a major concern as quality of cotton will be badly hit due to this single reason.

Based upon our surveys, we suggest that:

1. Diversity of *Bt-Cry* genes has to be maintained under a timeframe for durable resistance and to enhance the life of *Bt* transformed cotton varieties. Repeated use of only one *Bt* gene may result in the development of cross resistance in insect pests over a period of time. Expression of *Bt-Cry* genes in the approved cotton varieties need to be continuously monitored during the crop growing season and over the years according to international standards (1.5µg/mg). A threshold level of *Bt* toxin "*Cry*-protein" is very crucial as extremely low level of toxin may lead to the development of cross resistance.

2. R&D sector should be encouraged to transfer *Bt-Cry* genes into CLCuV resistant genetic backgrounds. The genotypes should be suitable for a given ecology and finally meet the set fiber quality parameters (fiber length and strength, GOT% etc.) and other desirable features required for the release of a normal commercial variety.

3. The capacity building of various agencies (FSC &RD, IPO, EPA/NBC & MinFA) is needed to facilitate the release of GM crops in the country.

4. There is also need of enough resources to cure the menace of spread of un-approved varieties which is illegal and damaging the cotton production, spread of new diseases and above all image of the country. The legal release of *Bt* cotton will be highly fruitful to tackle these issues and bring country in the list of legally GM crops adopting countries

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Table 4. ImmunoStrip analysis of samples collected from 48 locations of Sindh and Punjab in 2009-10.

S. No.	Genotype	Source	*Bt* genes expression intensity					
			Cry1Ac		*Cry2Ab*		Cry1F	
			1	2	1	2	1	2
1.	Local *Bt*	Shaheed Benazir Bhuttoabad	++	+++	-	-	-	-
2.	Aus-*Bt*	Shaheed Benazir Bhuttoabad	++	+++	-	-	-	-
3.	Bt-109	Shaheed Benazir Bhuttoabad	+	+	-	-	-	-
4.	Local-*Bt*	Shaheed Benazir Bhuttoabad	++	++	-	-	-	-
5.	Local *Bt*	Shaheed Benazir Bhuttoabad	++	++	-	-	-	-
6.	*Bt* 109	Shaheed Benazir Bhuttoabad	++	++	-	-	-	-
7.	Local-*Bt*		++	++	-	-	-	-
8.	Bt-703	Sanghar	+	+	-	-	-	-
9.	Aus-*Bt*	Sanghar	++	++	-	-	-	-
10.	Aus-*Bt*	Sanghar	++	-	-	-	-	-
11.	Local-*Bt*	Sanghar	+++	+++	-	-	-	-
12.	Local-*Bt*	Sanghar	++	++	-	-	-	-
13.	Aus-*Bt*	Sanghar	++	-	-	-	-	-
14.	Local-*Bt*	Sanghar	+	-	-	-	-	-
15.	Local-*Bt*	Sanghar	+++	-	-	-	-	-
16.	Local-*Bt*	Sanghar	+++	-	-	-	-	-
17.	Local-*Bt*	Mir Pur Khas	++	++	-	-	-	-
18.	Local-*Bt*	Mir Pur Khas	+++	+++	-	-	-	-
19.	Local-*Bt*	Mir Pur Khas	+	-	-	-	-	-
20.	Local-*Bt*	Mir Pur Khas	+++	+++	-	-	-	-
21.	Local-*Bt*	Mir Pur Khas	-	-	-	-	-	-
22.	IR-2389	Multan	+++	+++	-	-	-	-
23.	*Bt*-121	Multan	+++	++	-	-	-	-
24.	*Bt*-121	Multan	++	++	-	-	-	-
25.	IR-901	Multan	++	++-	-	-	-	-
26.	Bt-802	Multan	+	+	-	-	-	-
27.	Bt-802	Multan	++	++	-	-	-	-
28.	*Bt*-196	Multan	+++	+++	-	-	-	-
29.	CP 140	Multan	+	-	-	-	-	-
30.	*Bt*-196	Multan	+++	+++	-	-	-	-
31.	ASR-10	Multan	+++	-	-	-	-	-
32.	IR-2403	Multan	++	+	-	-	-	-
33.	ASR-3	Khanewal	+++	-	-	-	-	-
34.	*Bt*-448\8	Khanewal	-	++-	-	-	-	-
35.	IR-901	Khanewal	+++	+++	-	-	-	-
36.	NIBGE-I	Khanewal	+	-	-	-	-	-
37.	IR-1573	Khanewal	++	++	-	-	-	-
38.	Bt121	Khanewal	+++	+++	-	-	-	-
39.	Bt121	Khanewal	+++	+++	-	-	-	-
40.	BT121	Vehari	+++	+++	-	-	-	-
41.	ASR-3	Vehari	++	+	-	-	-	-
42.	*Bt*-133	Vehari	+	+	-	-	-	-
43.	IR-901	Vehari	++	++	-	-	-	-
44.	IR-1000	Vehari	++	++	-	-	-	-
45.	MG-2	Vehari	++	++	-	-	-	-
46.	ASR-12	Vehari	++	++	-	-	-	-
47.	ASR-7	Vehari	++	++	-	-	-	-
48.	MG-3	Vehari	++	++	-	-	-	-

+++ = Expression within 5 minutes, ++ = Expression within 15 minutes, + = Expression within 30 minutes, - = Lack of expression

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