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Full Length Article

A Temporal Expression of Cry1Ac Protein in Cotton Plant and its Impact on Soil Health

Muhammad Zaman[1], Muhammad Sajjad Mirza[2], Samra Irem[1], Yusuf Zafar[3] and Mehboob-ur-Rahman[1*]
[1]*Plant Genomics & Molecular Breeding (PGMB) Lab, National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan*
[2]*Deputy Chief Scientist, Soil and Environmental Biotechnology Division (SEBD), NIBGE, Faisalabad, Pakistan*
[3]*Minister (Technical), Permanent Mission of Pakistan to the IAEA Hofzeile 13, A-1190 Vienna, Austria*
[*]For correspondence: mehboob_pbd@yahoo.com; mehboob@nibge.org

Abstract

Like many other cotton growing countries, area under the cultivation of genetically modified cotton containing *Cry1Ac* gene has been increased tremendously in Pakistan because of its added advantage towards conferring resistance to bollworms especially *Helicoverpa armigera*. It has been reported that this gene derived from Bacillus thuringiensis has no negative impact on the soil ecosystem. Studies covering biosafety aspect of transgene involving Bt gene are non-existent in our ecological system. Every country has different microbiota which is the compelling factor to design studies for estimating the negative impact, if any, in our indigenous environment. In the present study, the concentration of Cry 1Ac protein in leaf tissues have shown increasing trend from 60 DAS to 90 DAS, and decreased at 120 DAS. Similar results were observed during each cropping season. Variation in protein concentration was found non-significant over different cropping years but it was significant at different sampling stages. We also observed that the Cry 1Ac toxin in the soil was stumpy low (78 times) when we compare it to leaf. Experiments related to the colony farming units (*CFUs*) of culturable bacteria, actinomycetes and fungi were undertaken in the rhizosphere of Bt- and non-Bt-cotton plants at various growth stages at 30 days interval from sowing till maturity for three consecutive years (2006-2008). A cotton variety IR-NIBGE-901 (containing *Cry 1Ac* gene, bred at NIBGE) and its non-Bt isogenic variety (FH-901) were sown in NIBGE cotton field, Faisalabad Pakistan. The concentration of Cry 1Ac protein released in the soil through root exudates, and in the terminal leaves was measured 60, 90 and 120 DAS. Results showed no significant impact ($P<0.05$) on CFUs of bacteria, actinomycetes and fungus between the Bt and non- Bt cotton rhizosphere during cropping season at one particular stage. However, the temporal and spatial variations for microbes were observed significant among the different time interval (30, 60, 90, 120, 150 and 180 DAS) and the year of cultivation. Thus, our studies have demonstrated that the repeated cultivation of Bt cotton had no significant impact on soil health of Pakistan.

Keywords: Bt cotton; Expression; Microbes; Soil health; Risk assessment

Introduction

Genetically modified crops containing *Bt* gene(s) have played a substantial role in sustaining crop yield, because of providing inbuilt protection against various insect pests, which not only reduces the use of pesticides but also helps in protecting our environment (Devare *et al*., 2004). Thus the technology can be an efficient part of integrated pest management system (Yu *et al*., 2011). Besides the positive impact of the cultivation of Bt crops—presently covered large area worldwide, there are environmental and biosafety concerns of the cultivation of Bt crops (Megha and Kaur, 2013).

Microbes, found abundantly in soil, play important role in in several processes in soil e.g. nutrient mineralization, organic matter breakdown, control plant pathogens, decay of agricultural chemicals, and soil structure improvement (Kapur *et al*., 2010; Han *et al*., 2013; Thabit and El-Naggar, 2013). Due to the close interaction of microbes and plants in the rhizosphere, there are chances of damaging soil microbiota by the release of Cry proteins (Kowalchuk *et al*., 2003). It is much more likely that Bt proteins can enter in soil through deposition of pollens, root exudates (Barriuso *et al*., 2012) and from the crop residues (Li *et al*., 2007). However, the eventual effects of Bt on soil biota depends on persistence and biological activity of Cry proteins in soil (Miethling-Graff *et al*., 2010).

Soil microorganisms can be exposed to Bt proteins because of the occurrence of root exudations or during the decomposition of Bt-plant in the soil as this phenomenon has been reported in Bt-corn containing *Cry1Ab* gene (Saxena *et al*., 1999; Stotzky, 2000). Some studies also confirmed the release of Bt protein in soil cultivated with Bt-cotton (Gupta *et al*., 2002).

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Multiple studies conducted to evaluate the impact of Bt crops on soil organisms, showed that Bt proteins have no harmful impact on the soil microbes even at far higher concentration of the Bt proteins. In a study, variations were not found in the soil microbiota of the fields with Bt plant material versus the fields with conventional plant material (Donegan *et al*., 1995, 1996). Soil macroorganisms including mites, collembola, earthworm, and snails did not show any harmful effect during laboratory and field experiments when exposed to Cry proteins (Honemann and Nentwig, 2009; Bai *et al*., 2010).

To examine the effects of Bt on soil microbiota, several studies failed to get any information related to considerable and undesirable impacts of Bt protein under laboratory and field conditions (Miethling-Graff *et al*., 2010; Tan *et al*., 2010) on both culturable and non-culturable population of microbes (Kapur *et al*., 2010).

In addition, any qualitative and quantitative change in the root exudates can robustly control the organization and the activity of rhizospheric microorganisms in case of Bt cotton (Yan *et al*., 2007). In soil, biodegradation of Bt protein is rendered due to its binding with clay particles and humic substances without deviating its prime activity to kill the larvae (Clark *et al.*, 2005; Saxena *et al*., 2010). It is reported that Bt protein cannot accumulate in soils for a long, as degradation of Bt protein in soil takes place quickly during initial stages of its entry in the soil and a little quantity can persist. (Li *et al*., 2007; Zurbrugg *et al*., 2010). Moreover, microbes are not getting affected from Bt protein directly, rather there are some other factors that contribute substantial role e.g. stage of plant growth, time period of straw decomposition, plant hybrid and variety (Yu *et al*., 2011). No considerable effect of Bt maize cultivation was observed on bacterial and fungal groups involved in transforming soil organic matter (Becker *et al*., 2014). Castaldini *et al*. (2005) reported difference in bacterial population, soil respiration, colonization and establishment of mycorrhizal in fields of Bt maize and non-Bt maize, however the risk was not well known.

The impact of Bt cotton was studied on different aspects of soil ecosystem e.g. microbes population, diversity and functions at different depths of soil grown with Bt and non Bt crop. High variation in microbial population and diversity index in soil was observed in case of hybrids cotton, different depths in the soils of Bt and non Bt cotton, and the amount of root exudates, distinct inherited properties of the plants instead of *Cry* gene (Velmourougane and Sahu, 2013) with no significant negative effects on processes of microbes. Hu *et al*. (2009) did not find any impact on the useful population of rhizospheric bacteria even after cultivating transgenic Bt cotton for several years.

Generally, research explored the impact of Bt on soil microbes and their enzymatic activity in the range of no to negligible or temporary effect but considerably can be attributed to the variation in geography, soil form, crop range, temperature, variation in chemical structure of Bt and non-Bt plants as low rate of soil respiration and their biomass respectively. Persistence levels of Cry protein were varied in some studies that may be directly related to microbial diversity of which indirectly rely on soil type, season, crop species, crop management practices other climate dependent factors that change with location and climate zone (Carpenter, 2011).

Soil ecosystem has also been changed at different crop growth stages (Wei *et al*., 2012). Furthermore the biological activity of soil can fluctuate by adding fertilizers along with farm yard manure. Intercropping of Bt crops with non Bt crops can facilitate in avoiding any harmful effect of Bt toxin on microbes (Singh *et al*., 2013b), although high density of microbial community was also observed in soil cultivated with transgenic cotton (Kapur *et al*., 2010).

In contrary to previous findings, an Indian report appeared which showed a significant reduction in count of actinobacteria (17%), bacterial (14%) and the activities of acid phosphatase (27%), phytase (18%), nitrogenase (23%) and dehydrogenase (12%) in the Bt- cotton fields versus non-Bt cotton fields. The amount of lignin because of Bt cultivation can also modify the intensity and role of decomposers in the soil (Xue *et al*., 2011). Similarly, change in soil microbial structure was observed in some studies. Hence, it is important to carry out extensive long-term studies to assess the Bt related risks on the populations of soil biota (Kapur *et al*., 2010). As Bt is soil borne microorganism, naturally produce Cry protein (roughly 0.25 g ha^{-1}) but increase in quantity is observed in farm soil (650 g ha^{-1}) in case of Bt cotton crop (Blackwood and Buyer, 2004). Harmful effect of Bt maize was observed on development and eggs hatchability of snails (Kramarz *et al*. 2009), but the hazard was not recognized due to limited information and requirement of further testing. Instead of crop itself, environmental settings and use of pesticide has utmost impact on diversity and uniformity of species as recommended by field observations (Birch *et al*., 2007; Cortet *et al*., 2007). There might be adverse impacts of Bt crops cultivation on indigenous microbial diversity of the soil (Giovannetti, 2003) and the chances of shift in the structure of native microbial population as a result of this inclusion (Bt protein with soil) may not be ruled out (Dunfield and Germida, 2004; LeBlanc *et al*., 2007). Further, the accumulation and degradation of Bt proteins may differ because of soil qualities like pH of that particular areas, like Australia, where cotton is cultivated on soils with pH ranging from 7.5–8.5 (Tapp and Stotzky, 1998), that helps in rapid degradation of Bt endotoxins by soil microorganisms. Pakistan is the 4th largest cotton producing country, where pH of the soil ranges from 8.5 to 9.5, supposing the degradation of Bt proteins rapidly.

Majority of the already reported studies and the included data from experiments, related to risk assessment of Bt crops have been carried out in pots and green houses that cannot be manipulated under field conditions due to limited soil ecosystem in pots. The cultivation of Bt crops

may cause variation in some aspects of microbes in rhizosphere with respect to seasonal and spatial changes.

More research is needed to realize the complicated system of plant-microbe interface thus leading towards improved understanding of ecological compatibility of genetically modified crops and to assess the impacts of continual farming of Bt crops on soil environment. The present study aimed to monitor the impact of Bt cotton cultivation on CFUs of microbes in Pakistan under field conditions. We monitored the soil for three successive years to evaluate the toxicity of transgenic Bt cotton on bacteria, actinomycetes and fungi at different growth stages of plants.

Materials and Methods

Experimental Site, Plant Material and Field Design

The experiment was carried out at the cotton field of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad Pakistan. This institute is situated 31°– 26'N latitude, 73°-06'E and at an elevation of 184.5 m from the sea level. The seed of non- transgenic cotton cultivar FH-901 was collected from the Cotton Research Institute (CRI), Ayub Agriculture Research Institute (AARI) Faisalabad. Transgenic genotype (IR-NIBGE-901) having the Bt source (Cry1Ac, Mon-531 event) was developed through a series of backcrosses by the Plant Genomics and Molecular Breeding (PGMB) Lab, NIBGE.

Both the (IR and Non IR) cotton cultivars were planted in randomized complete block design (RCBD) arrangement with three replications in normal cotton growing season (Kharif) for three consecutive years from 2006 to 2008. Each plot was having six rows with row length of 10 m and row spacing of 75 cm. Plant population density for each cultivar was 4.5 plants m^{-2}. The sowing was completed in the 1st week of June each year by drill method initially over seeded and then hand-thinned after the germination to maintain the plant to plant distance as 30 cm apart with in a row. Adequate number of irrigations were applied by flooding when necessary to eliminate any confounding effect of drought, especially during reproductive stage and the 4-5 days before soil sampling. Every year, the experimental area received 50 kg ha^{-1} of NPK in a pre-plant application and 50 kg ha^{-1} of N at flowering followed by 50 kg ha^{-1} of N at boll setting stage. Intensive management in cotton fields was carried out according to local agronomic practices unless otherwise indicated. Similarly, insect pest infestation was controlled by the application of insecticides.

Quantification of Cry1Ac Protein

Soil sampling: Every year, soil samples from the upper 20 cm soil layer were collected from experimental site for quantifying the presence of traces, if any, of Bt protein in the soil at 0 DAS. Rhizospheric soil (the soil fraction attached to the root) was collected for three consecutive years at 60, 90 and 120 DAS from Bt and non-Bt cotton fields. Soil sub-subsamples were collected from inner rows of each plot randomly. The plants were carefully dug out, and the soil adhering to their roots was collected in plastic bags. The sub-subsamples from each row were pooled into one composite sample and bring to laboratory for removing the visible plant materials. The samples were sieved and stored at -70°C until further use in ELISA.

Leaf sampling: For the quantification of Cry 1Ac protein, five to eight healthy plants from each replicated plot were selected. Terminal fully expanded leaves (upper fourth) were harvested from an individual plant per plot, placed in plastic bags, and transported to the laboratory in a container filled with ice. Within half an hour, three subsamples of two leaf disc were excised from each leaf by snapping the tube cap of 1.5 mL down on the leaf twice. Samples were weighed to determine the initial weight of leaf tissue (17-30 mg) and kept into the same 1.5 mL tubes and stored at -70°C until further use in ELISA.

ELISA: An enzyme linked immunosorbent assay (ELISA) Cry1Ab/Cry1Ac Plate kit, from Abraxis Warminster, PA was used to quantify the Cry 1Ac protein in soil extracts and leaf tissue from Bt and non-Bt cotton fields. This kit is designed for quantifying both Cry1Ab and Cry1Ac protein.

The kit was provided with 12 removable strips containing eight Cry 1Ab/Cry1Ac antibody coated wells each, strip holder, Cry1Ab/Cry1Ac antiserum solution, enzyme conjugate, color solution stop solution and purified Cry 1Ab standards (0.0 to 4.0 ng mL^{-1}). The dilution buffer and other kit components were kept at room temperature for 30 min before conducting assays.

Previously stored at -70°C and freshly collected leaf samples were utilized for measuring the Cry1Ac protein by adopting a procedure proposed by the manufacturer (Abraxis Warminster, Philadelphia, USA).

The tissue was ground by rotating the pestle against the sides of the tube with a twisting motion for about 30 s, or until the tissue was fully ground. In total, 0.5 mL of the 1 x sample extraction/dilution buffer was added in the tube. The issue was again crushed for 30 s to mix tissue with the extraction/dilution buffer. Tubes were kept for a few minutes. Before proceeding, 1:11 dilution (using Cry1Ab/Ac extraction buffer) was made. It is noteworthy that the contamination was avoided by using new extraction device and pestle for each plant sample.

For the stored soil samples, methodology was adapted as described by Rui *et al.* (2007). Briefly, a mixture containing 2 g of the soil and 3 mL of the extraction buffer (from the kit) was shaken for 10 m, extracted for 4 h and then centrifuged (16,000 × g) at 4°C. The amount of Cry1Ac protein in the supernatant was determined following the protocol supplied with (ELISA) kits by amending above steps.

The Abraxis (Warminster, Philadelphia, USA), specifically, was necessary to fit unknown Cry1Ab/Ac concentrations into the range of standards provided with the kit (0.0 to 4.0 ng mL^{-1}). Blank (0), Cry1Ab/Ac standards, the supernatants (diluted to be within the range of the standard concentration) were added to wells of ELISA plates to quantify the Cry protein following the manufacturer's protocol. Diluted Cry1Ac preparations and reagents were taken from vials and placed into PCR tube followed by transferring to ELISA strips. All the procedures for Cry 1Ac protein quantifications were replicated three times. Incubation and washing steps were done according to the manufacturer's instructions. The optical density was measured at 450 nm by a microplate reader. A standard curve was constructed by including purified Cry1Ab protein in the assay (0, 0.25, 0.5, 1, 2 and 4 ng mL^{-1}. A simple conversion was used to express values as "Cry1Ac" as dictated by the kit protocol. Each sample was quantified in triplicate, and the mean was calculated. The result of each sample was presented as ng Cry 1Ac protein per g of leaf tissue. For soil, the results were shown as ng Cry 1Ac per g of wet soil.

Survey of Rhizosphere Microbes by Plate Culture Method

Five plants from each of the cultivar were randomly selected on schedule dates (30, 60, 90, 120, 150 and 180 DAS), and were removed. About 60 g of rhizospheric soil was sampled from the removed plants. From this, 30 g was dried at 80°C for 1 day to obtain dry weight (DW), and the remaining 30 g of soil was mixed with 270 mL of 15 mM phosphate buffer (pH 7.0). The diluted soil solution (100 mL) was spread on two kinds of agar media, i.e. OGYE and PTYG media. OGYE medium, containing 5 g of yeast extract, 20 g of glucose, 0.1 mg of biotin, and 50 mg tetracycline per liter, was used for the incubation of fungi at room temperature. PTYG medium, containing 0.25 g of peptone, 0.25 g of tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 30 mg of $MgSO_4.7\ H_2O$, and 3.5 mg of $CaCl_2.2H_2O$ per liter, was used for cultivating actinomycetes and bacteria at room temperature. Three replicates of each medium were tested on five tagged plants from each genotype on all samples taken at 30, 60, 90, 120, 150 and 180 days of sowing (DAS). The colony numbers of fungi were counted at day 3, while those of actinomycetes and bacteria were counted at day 7.

Statistical Analysis

The community of bacteria including actinomycetes and fungi (log 10 CFU) was expressed as the means ± standard error using MS- Excel 2007. Analysis of variance (ANOVA) was used to find out the statistical difference between the data.

Results

Concentration of Cry 1Ac in soil under Bt Cotton Cultivation

The soil in which the plants had been growing were analyzed for Cry1Ac using a commercially available kit at different growth stages (60, 90 and 120 DAS) over three growing seasons (2006-2008). Results revealed that the Cry1Ac protein was not detected in any of the soil samples taken from the control plots cultivated with non-Bt cotton. There were no differences among the measuring concentration of Cry 1 Ac protein in the soil, where Bt cotton had been growing all the years (2006-2008), but significantly more ($P < 0.001$) Cry 1 Ac protein detectable in the soil at 90 DAS stage (Table 2). The highest concentrations of Cry 1Ac (3.603 ng g^{-1}) was found in the rhizosperic soil at 90 DAS in 2006, while the lowest (2.451 ng g^{-1}) was detected during 2008 (Fig. 1b) with an average concentration of 3.00 ng g^{-1} for all over the three years.

Concentration of Cry 1Ac in Leaf Tissues and Soil

Significant variations were found in concentration of Cry 1Ac protein in leaf tissues at growth stages (60, 90 and 120 DAS) and the years (2006, 2007 and 2008). Similarly the interaction among the years and growth stages was also significant (Table 2). The highest and the lowest concentrations of Cry 1Ac were found in leaf tissues at 90 and 120 DAS, respectively (291.05 ng g^{-1} and 150.5 ng $g^{-1,}$ respectively) during 2007 (Fig. 1a) with an average concentration of 235±5.19 for all over the three years.

Impact of Bt Cotton on Soil Microbes

Extracted CFUs of bacteria (including actinomycetes) and fungi from Bt and non-Bt soil were subjected to analysis of variance (ANOVA). There were no statistically significant differences ($P \geq 0.5$) in the CFUs of culturable bacteria (including actinomycetes) and fungi between rhizosphere soil of Bt and non-Bt cotton (Table 3). The only significant influence on the distribution of CFUs was the time of sampling (30, 60, 90, 120, 150 and 180 DAS). The CFUs of bacteria (including actinomycetes) and fungi were distinctly different at each of the sample stage for all over the three years ($P < 0.001$), irrespective of Bt trait (Fig. 2). During 2006 and 2007, bacterial and fungal population showed similar increasing trends in soil cultivated with Bt and non-Bt cotton between sampling time from 60-120 DAS and then decreased 150 and180 DAS. However, this pattern was non-significant and not observed in experiment during 2008 (Fig. 2).

Table 1: Physical and chemical characteristics of the 20 cm soil layer of the experimental site at NIBGE, Faisalabad, Pakistan

Characteristics	Unit	Mean
Physical		
Sand	% age	48.55
Silt	% age	23.45
Clay	% age	28.19
Organic matter	% age	0.81
Chemical		
EC_e	dS m^{-1}	1.42
pH		8.1
Organic Carbon	g kg^{-1}	3.65 ± 0.62
$CaCO_3$	g kg^{-1}	25.28 ± 2.12
Cation exchange capacity	$cmol_c$ kg^{-1}	6.35 ± 0.58
Total-N	g kg^{-1}	0.041 ± 0.01
$NaHCO_3$-P	mg kg^{-1}	6.90 ± 0.41
CH_3COONH_4-K	mg kg^{-1}	191 ± 6.89

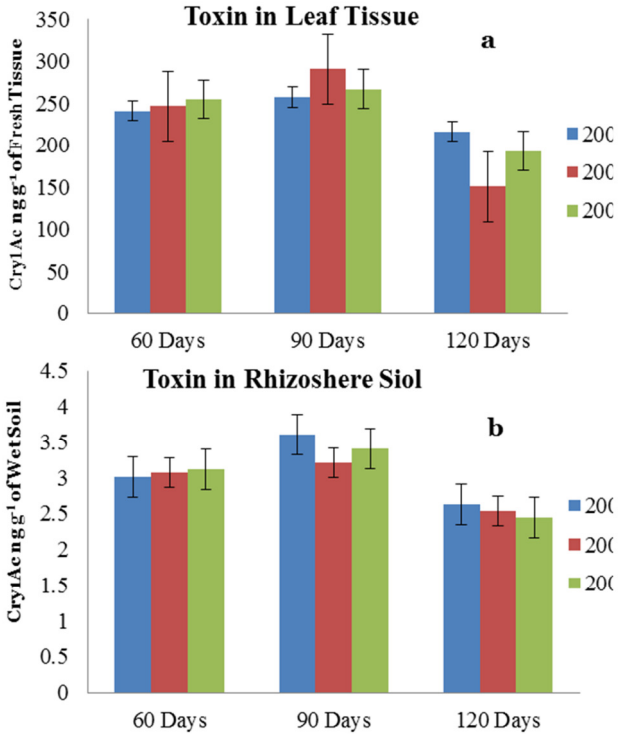

Fig. 1: Variation in Cry 1Ac toxin level in leaf (a) and rhizospheric soil (b) of IR-NIBGE-901 cotton during different growth stages (60, 90 and 120 DAS) from 2006-2008. Error bars indicate the standard error

Discussion

To assess the impacts of Bt transgenic crops, it is essential to measure the quantity of Bt toxin in the soil. For measuring the amount of Cry1Ac in rhizospheric soil, samples from fields with Bt and non-Bt cotton were collected in replicates. The results revealed that there were no traces of Cry 1Ac in soil from non-Bt fields, while the

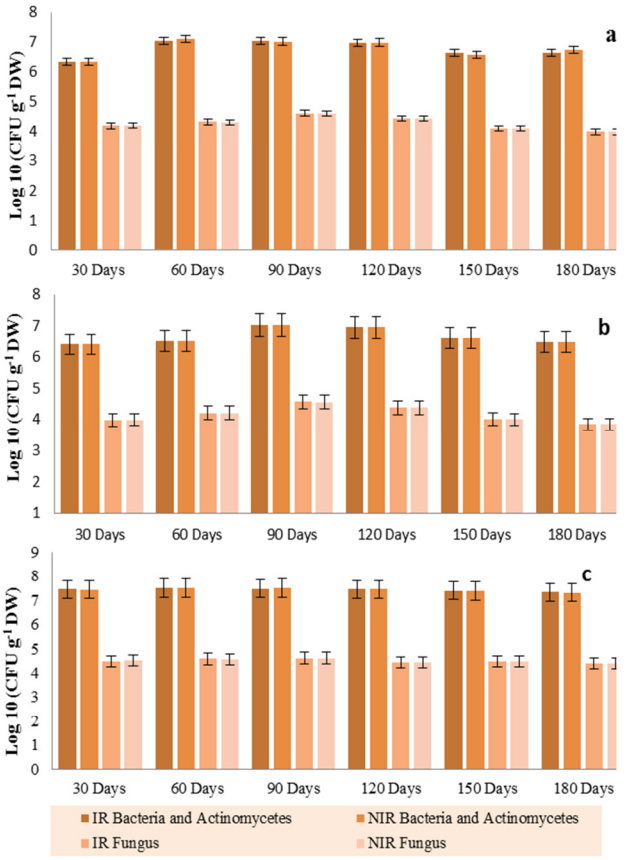

Fig. 2: Variation in Microbial population size (log10 of CFU) in rhizospheric soil of IR and NIR cotton during different growth stages in three replicates (a) 2006 (b) 2007 (c) 2008. Error bars indicate the standard deviation.

Cry 1Ac protein was detected in rhizospheric soil from fields under Bt cotton cultivation. The concentration of Cry 1Ac was in the range of 2.63 ng g^{-1} - 3.01 ng g^{-1} at different sampling times during first cropping season (2006) with average conc. of 3.00 ± 0.071 ng g^{-1} over all cropping years (Fig. 1b). The increasing trend in protein concentration from 60 DAS to 90 DAS, and decreased at 120 DAS are in the agreement with the findings of Helassa *et al.* (2011) that concentration of Bt protein declined over a period of 4 months. Furthermore it was reported that persistence of Bt toxin is dependent on physiochemical and biological properties of soil. Similar results were observed during each cropping season. Interestingly Cry 1 Ac was not detected in the soil of Bt cotton field at 0 DAS all the three years. Variation in protein concentration was found non-significant over different cropping years but it was significant at different sampling stages (Table 2). Decreasing trend was always observed at 120 DAS. Miethling-Graff *et al.* (2010) reported that no traces of Cry3Bb1was observed in the fields with non-transgenic maize and the concentration of Bt protein in rhizospheric soil decreases over time. Concentration of Cry1Ac was in range of 4.5-27.6 ng g^{-1} in

Table 2: ANOVA Table for Cry 1 Ac in Leaf Tissue and Soil Extract

Parameter	Source	df	ss	ms	*F* value	Probability	Significance
Cry1Ac in Leaf Tissue	Year	2	494.0	247.0	11.69	0.0213	S
	Error	4	84.5	21.1			
	Time (60, 90, and 120 DAS)	2	34688.4	17344.2	449.48	0.0000	S
	Year X Time (60, 90, and 120 DAS)	4	8286.2	2071.5	53.68	0.0000	S
	Error	12	463.0	38.6			
Cry1Ac in Soil Extract	Year	2	1.08923	0.54461	3.53	0.1307	Ns
	Error	4	0.61657	0.15414			
	Time (60, 90, and 120 DAS)	2	3.44041	1.72020	16.07	0.0004	S
	Year X Time (60, 90, and 120 DAS)	4	0.18579	0.04645	0.43	0.7818	Ns
	Error	12	1.28483	0.10707			

Table 3: Analysis of variance of CFUs of Bacteria (including actinomycetes) and fungus in IR-NIBGE-901 and NIR-FH-901 cotton

Year	Parameter	Sourec	df	ss	ms	*F* value	Probability	Significance
2006	Bacteria & Actinomycetec	Variety (IR & NIR)	1	1.37E+11	1.37E+11	4.482	0.102	Ns
		Error	4	1.22E+11	3.06E+10			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	5.89E+14	1.18E+14	1858.219	0.000	S
	Fungus	Variety (IR & NIR)	1	412254.1	412254.1	0.117	0.749	Ns
		Error	4	1E+07	4E+06			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	4E+09	9E+08	437.149	0.000	S
2007	Bacteria & Actinomycetec	Variety (IR & NIR)	1	1.77E+09	1.77E+09	0.024	0.884	Ns
		Error	4	2.94E+11	7.34E+10			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	4E+14	7E+13	1010.555	0.000	S
	Fungus	Variety (IR & NIR)	1	82.008	82.008	0	0.996	Ns
		Error	4	9E+06	2E+06			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	4E+09	8E+08	308.885	0.000	S
2008	Bacteria & Actinomycetec	Variety (IR & NIR)	1	3.33E+10	3.33E+10	1.391	0.304	Ns
		Error	4	9.57E+10	2.39E+10			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	3.56E+14	7.12E+13	312.308	0.000	S
	Fungus	Variety (IR & NIR)	1	7849.2	7849.2	0.009	0.93	Ns
		Error		4E+06	903652			
		Time (30, 60, 90, 120, 150, & 180 DAS)	5	4E+09	9E+08	684.305	0.000	S

soil of Bt cotton fields but it was found null in fields with non-transgenic isolines (Chen *et al*., 2012). It has also been reported that Bt protein cannot accumulate in the soils for a longer period of time, as degradation of Bt protein takes place quickly during initial stages of its entrance in the soil of which a little quantity can persist for a long time. (Li *et al*., 2007; Zurbrugg *et al*., 2010). In a study, Dubelman *et al*. (2005) reported that Cry 1Ab does not accumulate in the soil even after three successive years of Bt corn cultivation, which reinforce our results at pre-emergence stage i.e. 0 DAS of which Bt was not detected. The degradation of Bt protein in the soil is the matter of weeks (Sims and Holden, 1996) and has been enhanced in the soils having pH ranging from 7.5–8.5 (Tapp and Stotzky, 1998). Pakistan is the 4th largest cotton producing country, where pH of the soil ranges from 8.5 to 9.5 which suggest that there are meager chances of accumulation of Cry1Ac proteins in Pakistani soils as is also evident from the results of the present study.

Concentration of Cry 1Ac protein was higher in leaf tissues as compared to rhizospheric soil. The increasing and decreasing trend in concentration was in parallel to the pattern in rhizospheric soil. Maximum concentration was found in leaf tissues which was 291.05 ng g^{-1} during 2007 (Fig. 1a) with an average concentration of 235±5.19 for all over the three years, which is about 78 times more than the concentration in rhizospheric soil. Comparatively higher concentration of Cry1Ac protein was observed in leaf tissues. In another study, high concentration of Cry1Ab in MON 810 event Bt maize was found in leaf tissues (Monsanto, 2002; Nguyen and Jehle, 2007; Sze´ka´cs *et al*., 2010). Our results have exhibited a significant change in protein concentration in leaf tissues during different sampling time and also over the years. Pan *et al*. (2012) also reported that concentration of Cry1Ac protein increased gradually during the different growth stages of plant and starts decreasing at blossoming and boll forming stages, but the variation was non-significant. It was suggested that variation in Cry1Ac protein concentration may be due to prevailing environmental conditions.

As cultivation of Bt crops increased exponentially, concerns related to the biosafety of GM crops are also increasing. Risk assessment of GM crops in the actual field condition over longer period of time is needed to avoid any harmful impacts on local ecosystem. Impact of Bt cotton cultivation on populations of soil bacteria and fungi were studied at different growth stages of Bt cotton in field conditions for three consecutive years. Soil samples were analyzed of Bt and non-Bt cotton to evaluate the culturable microbial communities of soil. Although variation in microbial population was observed significant at different

growth stages at 5% probability (Table 3), but the variation pattern was statistically non-significant in bacterial population among soil from Bt and non-Bt cotton fields (p=0.05). During 2006 and 2007, bacterial and fungal population showed similar increasing trends in soil cultivated with Bt and non-Bt cotton between sampling time from 60-120 DAS and then a decreased 150 and 180 DAS. However, this pattern was non-significant and not observed in experiment during 2008 (Fig. 2). Soil samples collected from Bt cotton field showed similar results for CFUs of bacteria and fungi as compared to the results from non-Bt cotton fields.

Transgenic plants carrying *Bt* gene have revealed no undesirable impact on soil microbial community (Fang *et al*., 2012). In rhizospheric soil of Bt and non Bt cotton, non-significant variation was observed in population structure of various functional bacteria. Similar results were found even after growing Bt cotton over a longer period of time (Hu *et al*., 2009). Plantation of Bt transgenic crops over long periods of time do not have any significant changes on community structure of microbes, actinomycetes and fungi as well (Li *et al*., 2011). The results of the present study showed that Bt toxin released in the soil from various parts of the Bt cotton plants did not have any harmful effect on soil microbial community. Soils under cultivation of Bt and non-Bt cotton have not shown statistically significant variation among microbial population during incubation in all three replicates. Furthermore, no variation was observed in different experiments when cultivated Bt cotton on soil for three consecutive years. There were few deviations at various sampling periods but that was not constant from one sampling stage to the other. Such commonalities have been reported by studying the population of microbes and fungi in a soil cultivated with Bt corn (Saxena and Stotzky, 2001). Hu *et al*. (2013) suggested that cultivation of Bt cotton does not have any harmful impact on soil bacteria, actinomycetes and fungi. However, Singh *et al*. (2013b) reported that cultivation of Bt transgenic brinjal can cause a significant change in structure of soil microbial community during different growth stages of the crop but no difference was observed after post-harvest.

Change in microbial community during various growth stages may be attributed to variation in the nutrients e.g. carbon content of soil that may be one of the causative agents for change in microbial population. Significant negative impact of Bt cotton cultivation was observed as compared to pure peanut crop but that can be avoided by peanut intercropping and replacing urea with farm yard manure (Singh *et al*., 2013a). FlieÃŸbach *et al*. (2012) did not find any adverse effect of Bt maize on soil microbes even after repeated cultivation. It was suggested that Bt maize cultivation is safe to the microbial population. Han *et al*. (2013) investigated the impacts of growing Bt transgenic rice on population of soil microbes employing culture independent methods (Real-time PCR and PCR-DGGE). No significant impact was observed on bacterial, archaeal, fungal and functional microbial communities between Bt and non-Bt rice fields. Soils under Bt and non-Bt cotton cultivation showed significant variation in population of bacteria and fungi at 0-15 cm depth, while no variation was noticed at depth of 15-30 cm. Parallel mode was observed in case of yeast and actinomycetes. But no considerable adverse impact of increased microbial population was noticed on soil microbial activity in Bt cotton cultivated field as, no discrepancy was observed for useful microflora, at different soil depths. This variation may be because of difference in genetic makeup of cotton that cause change in amount of root exudates and root structural properties (Velmourougane and Sahu, 2013).

Conclusion

We concluded that the repeated cultivation of Bt cotton variety IR-NIBGE-901 for three consecutive years in the field has no adverse effect on CFUs of soil ecosystem. Moreover, Bt cotton caused detectable increases in the levels of Cry1Ac in rhizosphere soil. The differences detected by growing of Bt cotton were not as large as those resulting from seasonal changes both on microbial communities in the soil and Cry 1Ac concentration in plant and soil, showing that the effect of Bt cotton on soil ecology was within normal variation, as we expected in its conventional non-Bt variety (FH-901).

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