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A Case Study for Assessment of Microbial Community Dynamics in Genetically Modified Bt Cotton Crop Fields

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Abstract Bt cotton was the first genetically modified crop approved for use in India. However, only a few studies have been conducted to assess the feasibility of its commercial application. Bt cotton is genetically modified to express a proteinaceous endotoxin (Cry) encoded by *cry* gene of *Bacillus thuringiensis* that has specific insecticidal activity against bollworms. Therefore, the amount of pesticides used for growing Bt cotton is postulated to be considerably low as compared to their non-Bt counterparts. Alternatively, it is also speculated that application of a genetically modified crop may alter the bio-geochemical balance of the agriculture field(s). Microbial community composition and dynamics is an important descriptor for assessment of such alterations. In the present study, we have assessed the culturable and non-culturable microbial diversities in Bt cotton and non-Bt cotton soils to determine the ecological consequences of application of Bt cotton. The analyses of microbial community structures indicated that cropping of Bt cotton did not adversely affect the diversity of the microbial communities.

Introduction

Application of organochemical pesticides constitutes an important agricultural practice in developing countries such as India with agriculture based economies. However, with increasing awareness about the potential hazardous effects of these pesticides, several measures are being implemented to minimize their production and application. Development of insect/pest resistant genetically modified crops has been postulated as one such promising method [19, 21]. However, till date the use of genetically modified crops has remained nascent largely due to the lack of systematic information about ecological consequences associated with their release in the natural ecosystems [11, 15]. Bt crops (Bt cotton, Bt rice, Bt corn, etc.) are amongst the first genetically modified (GM) crops that were approved for research trial and commercialized use globally during the mid 1990s [9, 14]. The technology is specified by the generation of transgenic plants that express crystal protein endotoxin (Cry) encoded by the *cry* gene of the soil bacterium *Bacillus thuringiensis* having insecticidal activity against the common cotton infecting insects belonging to orders Lepidoptera, Diptera, and Coleoptera [12, 22].

The major advantage of the use of Bt crops is postulated to be the gradual reduction of residual pesticide load in agriculture fields with sustained or improved productivity (crop yield). On the other hand, it is also possible that cropping of Bt crops may induce adverse effect on native biological communities (including microbial diversity) of the fields used for cropping [11, 23]. Alteration of indigenous microbial community structure is postulated as one of the most likely consequences of incorporation of transgenic plant products in the soils [7, 13]. A few studies have indicated an undue reduction in the total microbial diversity during cropping of genetically modified crops [6, 13]. This undue reduction of microbial diversity is of significance and important concern since microbial diversity and richness is essential for maintenance of ecological stability and productivity of the environment. In light of the above rationale

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it is pertinent to systematically evaluate the microbial community structures and dynamics of the Bt and non-Bt cotton fields. Conventionally, the microbial community composition has been assessed by culture-dependent methods; however, these methods are selectively biased for enrichment of fast growing bacteria and usually allow the characterization of only a numerically minor fraction (usually less than 1%) of total microbial diversity [3, 4]. This limitation has been addressed by development of a number of culture-independent molecular approaches for analysis of total microbial community structures [3, 17, 20].

The present report describes a case study performed for evaluating the microbial community structure and dynamics of soil samples collected from Bt and non-Bt cotton fields at different times points (pre-cropping and post-harvest) during field trials. This report could serve to determine the feasibility of commercial application of Bt crops and other genetically modified crops.

Materials and Methods

Soil Samples and Site of Study

Soil samples for the study were provided by Monsanto India Ltd. from the trials conducted by National Centre for Integrated Pest Management (NCIPM), India. The samples were collected from Bt cotton and non-Bt cotton trial fields at Hotala village, located ~60 km away from Nanded in southern Maharashtra (77.7° to 78.15° East and 18.15° to 19.55° North). The amounts of pesticides used during different years varied in manner to sustain the optimal crop yields. For trials, two fields each (measuring ~20 m × 20 m in size) were cropped with Bt cotton and non-Bt cotton, respectively. These fields were designated as fields 1 and 2 for Bt cotton and fields 3 and 4 for non-Bt cotton, respectively. Soil samples were collected from 20 randomly selected spots (30 cm × 30 cm × 5 cm; each weighing ~300 g) within the trial fields for two cropping season (1st and 2nd season) at pre-planting (May, before the crops were planted) and post-harvest (December, after the crops had been harvested from the fields). Samples for respective fields and sampling time points were pooled together to make composite samples weighing ~6.00 kg, transported to the laboratory at 4°C and processed for further analysis. Samples were designated with field names viz., 1, 2, 3, and 4 and suffixed with 'a, b, c, d' for four sampling time points, respectively.

Transgenic Plants

Bt hybrid MECH-162 and non-Bt MECH-162 were planted in a total area of about 18 acres (including the trial fields). NHH-44, a *Gossypium hirsutum* hybrid was also planted as a popular check. Plantation was done according to the practices recommended by Integrated Pest Management (IPM) and as described by Bambawale et al. [2]. Under IPM practices maize plants and cowpea plant were sown along the borders of cotton fields to promote and preserve the activities of predators and parasitoids. One row of *Setaria* was also grown in between every 9th and 10th row of cotton as an attractant of insect predatory birds as an alternative measure for minimizing boll worm mediated damage.

Microbial Community Structure Analysis

Total Colony Forming Units (CFUs) in Soils

For determining CFU counts of bacteria, serial dilutions of soil samples were plated on various media viz., soyabean casein digest agar (SCDA), tryptone soya agar (TSA), and TSA containing antibiotics (0.1 mg/ml cycloheximide and 25 μg/ml amphotericin B). SCDA (composition in g/l: pancreatic digest of casein 15.0; papaic digest of soyabean meal 5.0; sodium chloride 5.0, agar 15.0; and pH at 25°C ~7.3) is a widely used medium, which supports the growth of wide variety of microorganisms. TSA (composition in g/l: pancreatic digest of casein 17.0; papaic digest of soyabean meal 3.0; sodium chloride 5.0; dextrose 2.50; dibasic potassium phosphate 2.50 and pH ~7.3 at 25°C) is a highly nutritious medium used for cultivation of a wide variety of organisms. In this medium, the casein digest and soyabean digest provide readily available amino acids and long chain peptides, while dextrose serve as the carbohydrate source. The dibasic potassium phosphate and sodium chloride contribute for buffering and maintenance of medium osmotic balance, respectively. Addition of cyclohexamide and amphotericin b to the above media was used to prevent fungal contaminations. For determining CFUs of fungi and actinomycetes soil dilutions were plated on potato dextrose agar (PDA) and actinomycetes isolation agar (AIA), respectively. PDA (composition g/l: potatoes infusion 200.00; dextrose 20.00; agar 15.00 and pH ~5.6 at 25°C) is a commonly used medium for growth of fungi, yeast and moulds. AIA (composition g/l: sodium caseinate 2.0; asparagine 0.1; sodium propionate 4.0; dipotassium phosphate 0.5; magnesium sulfate 0.1; ferrous sulfate 0.001; agar 15.0 g and pH ~8.1 at 25°C) prepared with 0.5% glycerol is used for isolation and cultivation of actinomycetes from soil and water samples.

Serial dilutions plating was carried out with 10^{-4} and 10^{-5} dilutions according to the standard procedure; briefly 5 g of air dried composite soil sample was re-suspended in 50 ml of 1× phosphate buffer saline (PBS) by thorough vortexing. After vortexing, the samples were allowed to stand for 15–30 min and re-suspension supernatants were collected for further dilution with PBS. Plates were

incubated under both aerobic and anaerobic conditions at 20 and 30°C for 2–6 days. (Incubations were performed in triplicates for each combination of dilution, aeration, and incubation temperature). The CFUs counting was performed at 2–3 days for aerobic incubations, while longer incubation times (5–6 days) were used for the anaerobic incubations. Morphologically distinct CFUs were enumerated and subjected to further identification. Noticeably, incubations carried out at 30°C yielded maximum number of morphologically distinct CFUs for different plating combinations.

Identification of Isolated Organisms

Morphologically different bacterial, fungal, and actinomycetes colonies obtained on various media were picked and patched onto fresh media to obtain pure cultures. The most predominant cultures obtained on plates were identified on the basis of morphological and biochemical tests and FAMEs analysis and taxonomically characterized according to Bergey's Manual of Systematic Bacteriology.

Terminal Fragment Length Polymorphism (T-RFLP) studies

Soil samples collected at different time points during field trials were subjected to T-RFLP analysis for determining the total microbial community structure and dynamics. Total soil DNA was isolated using UltraClean Mega Prep Soil DNA Kit (MoBio Laboratories, Inc., USA) according to the manufacturer's instructions. 16S rRNA gene pool of the total soil microbial community was PCR amplified with 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 926r (5′-TCA ATT CCT TTR AGT TT-3′) primers labelled with IRDye700^{TM} (infrared dye 700) and IRDye800^{TM} (Infrared Dye 800), respectively [16]. PCR reactions were performed in triplicates on Mastercycler Gradient (Eppendorf, Germany). The reaction cocktail contained 100–150 ng of total soil DNA, 0.5 units of Deep Vent DNA Polymerase (NewEngland BioLabs Inc., MA, USA), 1× ThermoPol Buffer, 200 µM dNTPs, and 100 pmol of each primer in a final volume of 50 µl. The reaction conditions were as follows: initial denaturation and enzyme activation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 75°C for 2 min. Final extension was performed at 75°C for 10 min. 16S amplicons were analysed on 1% agarose gel. Amplicons from replicate reactions were pooled together and precipitated according to the standard protocol. The precipitated samples were re-suspended in 20 µl of sterile deionised water and subjected to restriction digestion separately with *Hae*III and *Msp*I [5, 8] for 3 h in a reaction mixture containing 10 µl of re-suspended PCR product(s), 5 units of restriction enzyme and appropriate buffer as recommended by manufacturer (NewEngland Biolabs Inc.) [16]. Digestion product(s) were ethanol precipitated, ethanol washed, vacuum dried and carefully dissolved in 6 µl of TE buffer. Later, the dissolved samples were mixed with 4.5 µl of loading buffer consisting of 97.5% de-ionized formamide, 2.5% 0.5 M EDTA and 0.3% acidic fuchsine red. This mixture was denatured at 95°C for 3 min and immediately snap-cooled on ice for 5 min. One µl of denatured sample(s) was loaded on polyacrylamide gel (gel length: 25 cm) for electrophoresis in the LiCor4200L sequencing apparatus. Electrophoresis was carried out at 1,500 Volts in 1× TBE buffer (Tris base 890 mM, Boric acid 890 mM, and EDTA 20 mM; pH 8.3) for 1.5 h.

Calculation of Microbial Diversity Indices

In order to evaluate the richness and evenness of microbial diversity within soil samples collected from Bt and non-Bt cotton fields, the diversity statistics were calculated from total CFU counts and percentage normalized T-RFLP profiles. The average number and height of the T-RFLP peaks in normalized profile(s) were used as a representation of the relative abundance of different ribotypes/phylotypes. The above analysis was carried out only with terminal restriction fragments (TRFs) falling within the size range of 94 bases and 827 bases. Further, Shannon-Weiner diversity index was calculated as $H = \sum (\rho i) (\log_2 \rho i)$ where ρ is the proportion of an individual peak height. Simpson's index of diversity was calculated as $1 - D$; where D (Simpson's index) was determined with the formula $D = \sum (n/N)^2$. The values of n and N represent number of organism of particular species and total number of organisms of all the species, respectively. The scale for D ranged from 0 to 1. Microbial diversity Evenness (E) was calculated from the Shannon–Weiner diversity function with the formula $E = H/H_{max}$, where $H_{max} = \log_2(S)$ and S represents the species richness (the number of different species identified in the individual sample).

Multivariate Statistical Analysis

The T-RFLP profiles generated with different soil samples were subjected to 'Correspondence Analysis' (CA) using Multi Variate Statistical Package (MVSP); (Kovach Computing Services) for graphical representation of the dynamics of microbial community structure in different soil samples collected during field trial studies. The CA graph was plotted with 1st and 2nd Eigen values. In order to determine the possible relatedness of Bt cotton cropping or alteration in temperature/amount of rainfall on the dynamics of indigenous microbial community a 'Mantel Test' was performed on 'R' console (available from http://cran.r-project.org/bin/windows/rw-FAQ.html).

Chemicals

Cycloheximide and amphotericin B were obtained from Sigma Chemical Co. USA. Soyabean casein digest agar, tryptone soya agar, potato dextrose agar, actinomycetes isolation agar were procured from HiMedia Laboratories, Mumbai, India. The dyes labelled primers (for T-RFLP studies) were procured from MWG-Biotech Ebensburg, Germany. All other chemicals were of highest purity grade available locally.

Results

Microbial Community Structure Analysis

CFUs

In order to determine the culturable microbial diversity of the soil samples, total CFUs were determined. No significant difference was observed in the CFU count among Bt and non-Bt cotton field soil samples (Table 1). The CFUs counts obtained with the Bt cotton crop field post-harvest soil samples were similar to those obtained with pre-planting soil sample. Importantly, nearly all the Bt cotton soil samples indicated that the CFUs count did not decrease with the application of Bt-cotton. Instead some of the samples showed a slight increase of CFUs with Bt cotton cropping. The counts were found to decrease significantly only in case of plating performed on TSA medium containing amphotericin b; wherein CFUs count reduced from $19.2 \pm 0.24 \times 10^5$ to $12.8 \pm 0.16 \times 10^5$ CFUs/g of soil during 1st season for Bt cotton field 2 (Table 1). Similar reduction in the CFUs count was also observed with a few samples from the non-Bt cotton crop field soil samples. During the 2nd cropping season, the CFUs count of non-Bt cotton field 3 soil sample plated on TSA + cyclohexamide reduced from $21.5 \pm 0.14 \times 10^5$ to $13.8 \pm 0.17 \times 10^5$ CFUs/g soil.

The biochemical tests, morphological tests, and FAMEs analysis indicated that a large number of the isolated bacterial colonies belonged to genera *Bacillus* and *Pseudomonas* (data not shown). Dominance of these genera was similar in all samples of both Bt and non-Bt soils during the two cropping seasons.

Table 1 CFU counts in Bt and non-Bt cotton soil samples collected at different sampling seasons

Media	Bt soils							
	1st season pre-planting		1st season post-harvest		2nd season pre-planting		2nd season post-harvest	
	1a	2a	1b	2b	1c	2c	1d	2d
SCDA	5.2 ± 0.28	4.13 ± 0.19	4.9 ± 0.21	5.1 ± 0.20	6.1 ± 0.32	4.82 ± 0.25	5.93 ± 0.28	4.97 ± 0.21
TSA	30.2 ± 0.54	13.6 ± 0.19	27.8 ± 0.28	14.4 ± 0.21	14.2 ± 0.26	12.0 ± 0.22	14.72 ± 0.19	13.8 ± 0.11
TSA + Cyc	22.4 ± 0.31	18.2 ± 0.26	21.7 ± 0.12	19.3 ± 0.16	20.1 ± 0.19	19.2 ± 0.17	22.6 ± 0.20	19.5 ± 0.24
TSA + Amph	17.2 ± 0.20	19.2 ± 0.24	15.0 ± 0.14	12.8 ± 0.16	19.5 ± 0.15	20.2 ± 0.11	18.8 ± 0.17	21.2 ± 0.24
TSA (Anaerobic)	2.1 ± 0.04	1.9 ± 0.05	2.5 ± 0.04	1.8 ± 0.08	0.7 ± 0.03	0.62 ± 0.01	0.6 ± 0.01	0.9 ± 0.04
PDA	6.2 ± 0.24	7.2 ± 0.19	6.8 ± 0.14	7.8 ± 0.13	10.0 ± 0.16	7.4 ± 0.19	9.7 ± 0.16	8.4 ± 0.21
AIA	4.2 ± 0.12	6.8 ± 0.11	5.4 ± 0.17	7.1 ± 0.12	12.6 ± 0.12	13.7 ± 0.16	12.4 ± 0.16	13.4 ± 0.10
Media	Non-Bt soils							
	1st season pre-planting		1st season post-harvest		2nd season pre-planting		2nd season post-harvest	
	3a	4a	3b	4b	3c	4c	3d	4d
SCDA	4.2 ± 0.18	5.3 ± 0.16	4.3 ± 0.17	4.9 ± 0.20	5.2 ± 0.22	5.6 ± 0.18	6.1 ± 0.17	5.5 ± 0.14
TSA	10.5 ± 0.24	15.8 ± 0.28	12.2 ± 0.19	15.2 ± 0.21	11.1 ± 0.18	12.0 ± 0.22	8.2 ± 0.19	10.8 ± 0.11
TSA + Cyc	12.3 ± 0.21	16.2 ± 0.16	13.1 ± 0.16	15.3 ± 0.14	21.5 ± 0.14	17.2 ± 0.17	13.8 ± 0.17	18.5 ± 0.24
TSA + Amph	14.1 ± 0.20	16.2 ± 0.23	13.8 ± 0.14	15.9 ± 0.13	11.4 ± 0.19	12.2 ± 0.14	12.2 ± 0.20	13.1 ± 0.24
TSA (Anaerobic)	2.4 ± 0.04	1.9 ± 0.051	2.0 ± 0.03	1.2 ± 0.01	0.7 ± 0.03	0.62 ± 0.01	0.6 ± 0.01	0.7 ± 0.04
PDA	5.0 ± 0.18	6.9 ± 0.16	4.8 ± 0.14	3.9 ± 0.13	10.4 ± 0.16	8.4 ± 0.19	9.6 ± 0.16	8.0 ± 0.21
AIA	5.1 ± 0.12	6.8 ± 0.11	5.4 ± 0.17	3.6 ± 0.12	12.6 ± 0.12	13.7 ± 0.16	12.4 ± 0.16	11.4 ± 0.10

The counting was performed in triplicates and the average arithmetic values are presented. Values are presented as $\times 10^5$/gm of soil determined for triplicate experiment. Data obtained were analysed with Student's *t* test. *P* values ≤ 0.05 were considered statistically significant

SCDA soyabean casein digest agar, *TSA* tryptone soya agar, *Cyc* cyclohexamide, *Amph* Amphotericin b, *PDA* potato dextrose agar, *AIA* actinomycetes isolation agar

T-RFLP Analysis

In order to determine the dynamics of non-culturable microbial diversity of soil samples from Bt and non-Bt cotton fields at pre-planting and post-harvest stages, T-RFLP analysis was performed using 16S rRNA gene as the target phylogenetic marker gene. Comparative analysis of the terminal restriction fragments (T-RFs) and their relative intensities indicated only subtle changes in the microbial community composition (Supplementary Material, Figure S1). Figure S1 shows representative T-RFLP profile comparison of Bt cotton field 1 and non-Bt cotton field 3 at pre-planting and post-harvest during 1st and 2nd cropping seasons. The results obtained with T-RFLP analysis showed variation in band intensity (relative abundance) of only a few T-RFs (100, 101, 170, 219, 228, 234, 253, 262, 298, 309, 312, and 320 base pair lengths) among the different soil samples (Supplementary Material, Figure S2). Variations observed with these T-RFs were found to be statistically significant. However, variation in the relative abundance of these T-RFs was not observed to follow a diversity decrease relationship with the use of Bt-cotton for cropping. In order to further ascertain this observation, the T-RFLP profile data was subjected to correspondence analysis (CA).

Correspondence Analysis

CA is a statistical visualization method for graphical representation showing the degree of association/similarity among samples presented in the form of a two-way contingency table. This analysis is especially useful when each sample point (e.g., total microbial community structure) is explained by a large number of variables [1]. CA analysis compresses the variables to yield few Eigen axes each explaining the percentage significance for distribution pattern of sample points in the resultant graph [1, 10]. The distance among sample points on the CA graph signifies the dissimilarities, whereas their close clustering indicates similarity of the sample points. During the present study, the T-RFLP profiles for Bt cotton and non-Bt cotton soils from pre-planting and post-harvesting time points were taken as sample points and each T-RF (band) was explaining variables. The CA results were obtained in the form of Eigen Axis 1 and Eigen Axis 2 defined by 34.6% and 27.8% significance, respectively. The CA plot generated with axes 1 and 2 was statistically significant (together representing 62.4% relatedness of sample points) and positioned all the soil samples in a closely placed cluster (Fig. 1). Phylotype richness(s) was calculated with the total number of distinct

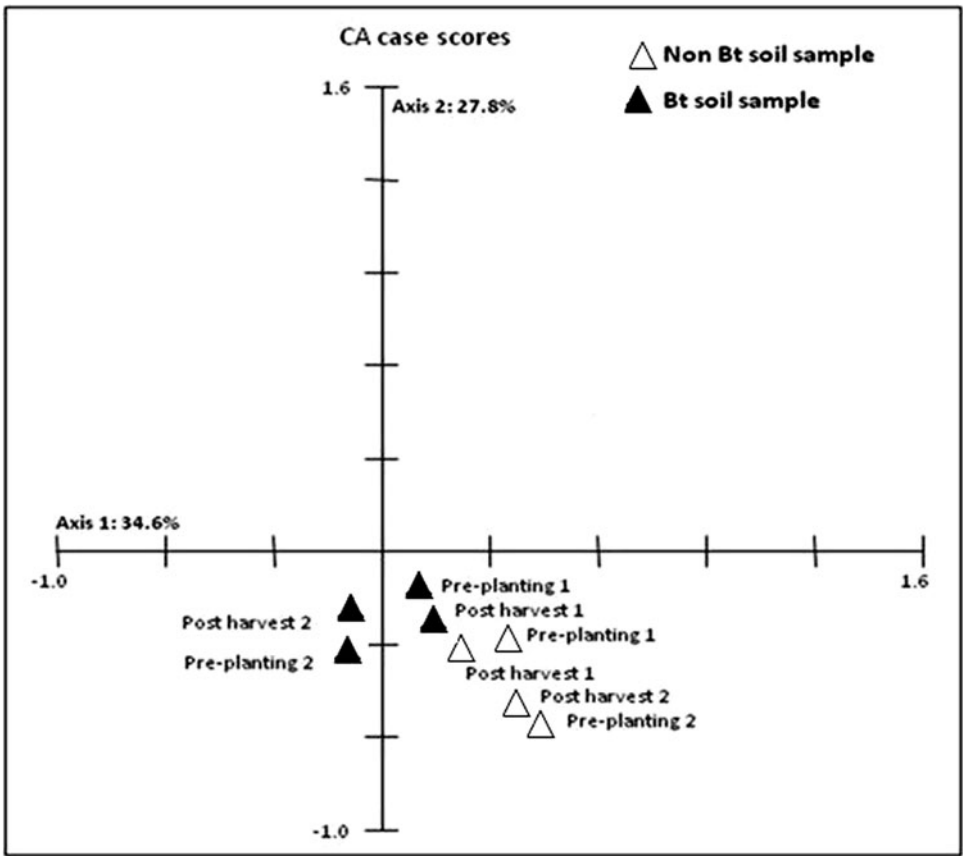

Fig. 1 Correspondence analysis (CA) plot generated with axis 1 and axis 2 that represented >60% of the relative microbial community structure. The positioning of total microbial community structures in different soil samples were observed to be closely clustered indicating stable microbial community structure

Table 2 Community richness and diversity analysis of the soil samples from Bt and non-Bt cotton fields

	Pre-planting		Post-harvest	
	Non-Bt	Bt	Non-Bt	Bt
S	39	34	38	33
H_1	4.49	4.26	4.21	4.09
$H_{1\max}$	5.28	5.08	5.24	5.04
$1 - D$	0.14	0.16	0.19	0.18
Evenness	85.03	83.86	80.30	81.24

Diversity indices were calculated on the basis of T-RFLP data taking the % relative intensities of different bands into consideration

S Phylotype richness, H_1 Shannon–Weiner diversity index, $1 - D$ Simpson's index of diversity

TRF sizes (between 200 and 403 bp) in all the T-RFLP profiles. Table 2 shows a summarized result of species richness calculated as 'Phylotype richness', 'Shannon–Weiner diversity index' and 'Simpson index of diversity' at pre-planting and post-harvest time points of two cropping seasons. These results were in close agreement with those obtained with CA analysis and indicated that species richness did not vary significantly within Bt and non-Bt soils. The other important statistical value viz., Simpson's index of diversity was also found to have closely associated values (Table 2).

Mantel Test

The temperature and rainfall patterns were used as variables for explaining minor changes in microbial community structure with the help of 'mantel test' analysis. The result obtained from this analysis (shown by a low mantel test score $\sim$78.5%) revealed that minor changes in the bacterial community are independent of the weather differences.

Discussion

Application of pest resistant genetically modified crops, e.g., Bt cotton has been postulated as one of the most effective means for minimizing the pest mediated damage to economically important crops as well as for reducing the use of toxic chemical pesticides [9, 18]. In the present study, the microbial community dynamics and structure of the Bt soils were compared to the non-Bt soils to assess the ecological consequences of cropping Bt cotton. In the present case, contrary to earlier reports that have indicated undue decrease in microbial community richness with the use of genetically modified crops [7, 13], the relative intensity of most of the varying T-RFs (170, 219, 228, 253, 262, 309, and 320) showed a significant increase. The remaining bands within T-RFLP profiles of Bt and non-Bt cotton fields were found to be quite stable (Supplementary Material, Figure S2). In order to further corroborate the stability of microbial community structures in different soil samples the T-RFLP profiles were subjected to CA analysis. The microbial community structures clustered according to the test field instead of the Bt or non-Bt cotton crop treatments. Soil samples of pre-planting and post-harvest time points from Bt cotton field 1 clustered together, similarly those from Bt cotton field 2 clustered together. The results obtained with CA analysis further proved that the microbial community structure in agriculture fields was not affected by the cropping of Bt cotton and the total microbial community structures of test fields remain quite similar during the cropping of both Bt cotton and non-Bt cotton.

Bt soil samples were observed to have almost similar microbial community diversity (richness and evenness) in pre-planting and post-harvest samples (Table 2). The culturable microbial diversity was also observed to be quite stable. A few minor variations observed in case of microbial community structures could have occurred because of the influence of environmental factors, e.g., seasonal variation in environmental factors. Therefore, the basic data collected for temperature profiles and rainfall patterns were used for determining their impact on microbial community composition and their dynamics during field trials. The field trial sites had a maximum temperature ranging from 26 to 37°C whereas minimum temperature was in the range of 19–22°C. Higher temperatures were usually observed during pre-planting time points, whereas, post-harvest time points experienced lower temperatures. The rainfall patterns were characterized by semi-arid climate having very little rainfall; maximum rain of $\sim$170 and 145 mm week^{-1} was observed during 1st and 2nd season, respectively. However, mantel test analysis clearly demonstrated that the bacterial community is independent of the weather differences.

Conclusion

To conclude, in the present case study analysis of microbial community structure dynamics indicated that Bt cotton did not cause adverse effects on the culturable as well as non-culturable microbial diversity. The microbial community structures were stable for phylotype diversity richness and evenness during field trial studies. Only a few subtle variations were observed within the microbial community structure that were not found to be directly associated with the cropping of Bt cotton. These results clearly demonstrate the selective advantage of using Bt cotton and indicate the possibility of ecologically safe application of genetically modified crops, e.g., Bt cotton.

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