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Assessment of the Influence of Transgenic Cotton on Beneficial Soil Rhizosphere Microbes

Sivaji Mathivanan[1*] and Sridar Raghupathi[2]

[1]Sugarcane Breeding Institute (ICAR), 641007, Coimbatore, India.
[2]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, 641003, Coimbatore,India.

Authors' contributions

This work was carried out in collaboration between both authors. Author SR was responsible for study design and supervision of work. Author SM was responsible for literature searches, laboratory work, data analysis and manuscript preparation. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

In this study the impact of Bt cotton has been accessed on the population of beneficial microbes in the rhizosphere of transgenic Bt cotton line (RCH-2 BG II) and its counterpart (a non-Bt cotton line). The *Bacillus thuringiensis* (Bt) proteins i.e., Cry1Ac and Cry2Ab were released from root exudates of Bt cotton (RCH-2 BG II) was confirmed by qualitative Enzyme-Linked Immunosorbent Assay (ELISA). Rhizosphere soil samples were collected in fifteen days interval i.e., 15, 30, 45 and 60 days after sowing (DAS). *Azospirillum brasilense*, *Bacillus megaterium*, *Pseudomonas fluorescens* population was more on 15th day in non Bt rhizosphere than Bt except *Trichoderma viride,* where as in 45th and 60th day all four microbes were more in Bt cotton (RCH-2 BG II) rhizosphere than its counterpart RCH-2. The statistical analysis of the data obtained from the pot cuture showed no significant differences in the numbers of CFU of *Azospirillum brasilense*, *Bacillus megaterium* (biofertilizer), *Pseudomonas fluorescens* and *Trichoderma viride* (biocontrol agents) between rhizosphere soil of Bt and non-Bt cotton. Restriction enzyme analysis of 16S rRNA of *Azospirillum*

**Corresponding author: E-mail: sivajibt@gmail.com;*

brasilense, *Bacillus megaterium* (biofertilizer), *Pseudomonas fluorescens* and ITS region of *Trichoderma viride* obtained from Bt and non Bt cotton rhizosphere in different intervals showed similar banding pattern between them.

Keywords: Bt cotton RCH-2 BG II; Cry protein; ELISA; beneficial microbes; rhizosphere; Restriction enzymes.

1. INTRODUCTION

A transgenic approach to crop protection was realized in the mid 1990s with the commercial introduction of genetically modified insect-resistant crops. Field and laboratory studies showed that transgenic plants expressing *Bacillus thuringiensis* Berliner (Bt) Cry proteins afford effective resistance to the larvae of a number of Lepidoptera species. For example, Bt cotton plants are protected against the cotton bollworm *Helicoverpa armigera* (Hubner) Shelton et al. [1] Deng et al. [2], thus reducing the requirement for multiple insecticide treatments [3] and the risk of pollution from chemical insecticide applications.

According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), worldwide production of genetically modified crops has increased 67-fold, from 1.7 million ha in 1996 to 114.3 million ha in 2007 [4] and is predicted to increase even more in the future. In India nine million hectares (M ha) area was under cotton cultivation and it is one fourth of the global area under cotton cultivation (35 M ha). *Bacillus thuringiensis* (Bt) cotton was introduced in India in 2002. Following its success, during the last 7 years (2002-2008), the area under Bt cotton has increased by 7.6 M ha from 0.029 M ha [4]. Although there are diverse benefits of Bt cotton, public concern also exist because both *in vitro* and *in vivo* studies on Bt cotton showed that Bt toxin produced in leaves, stems and roots of Bt cotton plants is introduced in soil. Bt-toxin from Bt cotton plants introduced into the soil through two pathways, i.e., biomass incorporation and root exudates [5].

Several experiments were conducted to assess risk of Bt cotton on flora and fauna in diverse agro ecosystems [6,7]. Some studies indicate that Bt cotton has no negative effects on soil flora and fauna and may even have beneficial effects [5]. While some studies have reported that Bt cotton creates adverse effects [8,9]. However, several experiment has been done to find out impact of transgenic crop with culturable and unculturable microbes (whole microbiome) but experiments respect to beneficial microbes alone is very very limited. Therefore the study has been conducted to find out the effects of Bt cotton on population of selected four beneficial microbes and its functional activity under *in-vitro* conditions.

2. MATERIALS AND METHODS

2.1 Collection and Maintenance of Cultures

The cultures of *Azospirillum brasilense*, *Bacillus megaterium* (biofertiizers), *Pseudomonas fluorescens* and *Trichoderma viride* (biocontrol agents) recommended for cotton crop were collected from biofertilizer unit of Agricultural Microbiology and Plant Pathology department, Tamil Nadu Agricultural University, Tamil Nadu, India respectively for the present research. Additionally *R. solani* (plant pathogenic fungi) also collected from Pathology department to check the efficacy level of biocontrol agents against pathogen. Bacterial cultures were streaked on slants and kept in 4ºC for maintenance. *Trichoderma viride* and *R. solani* culture were maintained on PDA slants.

2.1.1 Antibiotics resistance of beneficial microbes

In this study six antibiotics were tested against four microbes *viz., Azospirillum brasilense, Bacillus megaterium, Pseudomonas fluorescens* and *Trichoderma viride* to find out the resistance level of beneficial microbes against the antibiotics by following paper disc method. Antibiotic solutions were prepared and filter sterilized by using 0.22 µm nitrocellulose filters.

2.2 Preparation of Inoculums and Seed Treatment

Pot culture study was carried out in green house condition. *Azospirillum brasilense, Pseudomonas fluorescens* and *Bacillus megaterium* were inoculated in Malic acid broth, LB broth and KB broth respectively, kept for incubation on

incubator cum shaker for 72 hours at 32ºC. *Trichoderma viride* discs are transferred to PDA broth and kept in room temperature for 72 hours. After three days the *T. viride* mat formed on the surface of the medium was mixed well and these microbial cultures were used to treat the RCH-2 (non Bt) and RCH-2 BG II (Bt cotton) seeds. Bt and non Bt cotton seeds were obtained from Rasi Seeds Pvt. Ltd., Attur, Tamil Nadu. Cotton seeds were surface sterilized for 2 min with 70% ethanol followed by 2% sodium hypochlorite (10 min) and rinsed in sterile distilled water. A quantity of 10 ml of culture was mixed with 0.2 g of carboxymethyl celluose (2%) two sets to treat Bt and non Bt cotton seeds. Seeds mixed with culture inoculums and kept for incubation for half an hour. After incubation treated seeds were shade dried for half an hour before sowing in pots.

2.3 Design and Treatments

Transgenic Bt cotton RCH-2 BG II and the non transgenic RCH-2 were used in all the experiments. Details of design followed, factors involved and number of treatments and replication for this study is given below,

Design : FCRD
No. of factors : 2 (Beneficial microbes and cotton varieties)
No. of treatments : 10 (5 Beneficial microbes combinations and 2 cotton varieties)
No. of replications : Three

The experiment was carried out under greenhouse condition and soil samples from the rhizosphere of Bt cotton and non Bt cotton plants were collected at regular interval (15 days). First Sampling was done 15 Days After Sowing (DAS), second sampling was at 30 DAS, third sampling was at 45 DAS and, fourth sampling was at 60 DAS. Details of the treatments are given in Table 1. Qualitative ELISA assay was done with DesiGen qualitative ELISA kit from 30 days old plants of non Bt (Treatments 1-5) and Bt (Treatments 6-10) cotton plant rhizosphere soil sample.

2.4 Estimation of Microbial Population

Rhizosphere soil samples were taken for 15, 30, 45 and 60 days and in all the treatments to enumerate the rhizosphere microbes (*Azospirillum brasilense, Pseudomonas fluorescens, Bacillus megaterium* and *Trichoderma viride*) population. The Colony Forming Units (CFU) of functional bacteria and fungi in each sample determined by spreading 100 µl of the 10 fold serially diluted rhizosphere soil samples on appropriate culture media on Petri plates. Colonies were counted visually and expressed as CFU g^{-1} dry soil.

Table 1. Details of different treatments

Factor 1 (Beneficial microbes)	Factor 2 (Cotton varieties)
B1 - No inoculation	C1 - RCH - 2 (non Bt)
B2 - *Azospirillum brasilense*	C2 - RCH - 2 Bollgard II
B3 - *Bacillus megaterium*	
B4 - *Pseudomonas fluorescens*	
B5 - *Trichoderma viride*	

2.5 Statistical Analysis

Factorial Anova was carried out to find out the interaction of bioinoculants, non Bt cotton and Bt cotton varieties with different intervals with 5% significance level in AGRESS software.

2.6 Molecular Analysis of the Functional Microbes

Four beneficial microbes collected from Bt and non Bt cotton rhizosphere, at four different stages from pot culture were chosen for the molecular analysis to find any variation among them. Total Genomic DNA was isolated from eight isolates each from Bt and non Bt rhizosphere of all three bacteria by following the protocol of Sambrook and Russell [10] with slight modification. The genomic DNA was isolated from *Trichoderma* isolates by following the protocol of Hegedas and Khazhatourians [11].

2.7 Amplification Structural Gene by PCR

2.7.1 Amplification 16S rRNA and ITS region from microbes

The total genomic DNA isolated from selected *A. brasilense, B. megaterium, P. fluorescens* was subjected to amplify 16S rRNA gene and ITS region from *T. viride* isolates by Polymerase Chain Reaction (PCR). PCR was carried out by using the Eppendorf Master Cycler, Gradient (Eppendorf, Germany) with the final volume of 25 µl reaction mixture. 25 µl reaction mixture

Table 2. Details of primers used for amplification of 16S rRNA and ITS region

Target gene	Primer	Primer sequence (5' – 3')	Reference
16S rRNA	27f	AGAGTTTGATCCTGGCTCAG	Marchesi et al. (1998) [26].
	1492r	ACGGYTACCTTGTTACGACTT	
ITS region	ITS 1	TCCGTAGGTGAACCTGCGG	White et al. (1990) [27].
	ITS 4	TCCTCCGCTTATTGATATGC	

Table 3. Components of reaction mixture for restriction analysis

S. No.	Components	*Hae*III restriction	*Alu*I restriction
1	PCR product	10.0 µl	10.0 µl
2	10X Assay buffer for RE	3.0 µl	3.0 µl
3	Restriction enzyme (10 unit)	1.0 µl	1.0 µl
4	Sterile water	16.0 µl	16.0 µl
5	Total Volume	30 .0 µl	30 .0 µl

contains Genomic DNA (50 ng) 1.0 µl, 10X Taq. assay buffer 2.5 µl, dNTP's mix (10 mM) 1.0 µl, Forward primer (10 pM) 1 µl, Reverse primer (10 pM) 1 µl, $MgCl_2$ 1.0 µl, Taq. DNA polymerase (0.3 U) 0.5 µl and Autoclaved milliQ water 17 µl. The details of primers used to amplify 16S ribosomal RNA (16S rRNA) and ITS region are given in Table 2. Steps followed in PCR are as follows. Amplification of 16S rRNA region Initial denaturation at 95℃ for 4 min, denaturation 94℃ 45 sec, annealing 55℃ 45 sec and extension 72℃ 1 min 30 sec 35 cycles and final extension 72℃ 10 min, where as to amplify ITS region of *T. viride* Initial denaturation at 95℃ for 4 min, denaturation 94℃ 45 sec (35 cycles), annealing 59℃ 45 sec(35 cycles) and extension 72℃ 45 sec (35 cycles) and final extension 72℃ 10 min.

2.7.2 PCR-RFLP analysis

PCR RFLP restriction analysis was carried out with the amplified 16S rRNA products of selected isolates of the functional microbes. Approximately 10 µl of PCR amplified products were restricted with endonucleases *Hae*III (Thermo scientific) and *Alu*I separately at 37℃ for 3 hours and resolved by electrophoresis in 1.5% agarose gels for about 50 min at 100 Volts. components of restriction reaction mixture is given in Table 3.

3. RESULTS

3.1 Effect of Bt Protein on Functional Microbes under Pot Culture

Population of four beneficial microorganisms (*A. brasilense*, *P. fluorescens, B. megaterium* and *T. viride*) were enumerated from Bt and non Bt cotton rhizosphere soil. This experiment was done to find out the effect of Cry proteins (Cry1Ac and Cry2Ab) on functional microbes. The samples extracted from rhizosphere soil of Bt cotton plants showed positive for the Elisa test by turning yellow colour in the ELISA plate wells whereas rhizosphere soil samples extracted from non Bt failed to turn the yellow colour (remaining colourless) showed no Cry protein present in the root exudates of non Bt cotton. The result clearly indicated that root exudates of Bt cotton rhizosphere contain Bt toxin.

The results of antibiotic resistance assay of the four microbes viz., *Azospirillum brasilense, Bacillus megaterium, Pseudomonas fluorescens* and *Trichoderma viride* against six antibiotics revealed that *Azospirillum brasilense* showed resistance to antibiotics, Ampicillin, Tetracycline and Penicillin at 200 ppm, 100 ppm and 50 ppm respectively. *Bacillus megaterium* was resistant to 30 ppm Penicillin and 100 ppm of Choloramphenicol, whereas *Pseudomonas fluorescens* was resistant to Rifampicin at 20 ppm. *Trichoderma viride* exhibited resistance to Penicillin (30 ppm) and Griseofulvin (100 ppm). Native soil population did not grow in the respective antibiotics at above mentioned concentrations.

Pot culture study was carried out in green house to study the effect of Bt protein (endotoxin) released in the rhizosphere of the Bt cotton on the functional microbes (*A. brasilense*, *P. fluorescens, B. megaterium* and *T. viride*) with different exposure time. The experimental results showed that *A. brasilense* recorded high population (50 $x10^6$ CFU. g^{-1}) in Bt cotton and 30 x 10^6 CFU. g^{-1} in non Bt cotton at 30 DAS. Except 15 DAS remaining all sampling times 30 DAS (50 x 10^6 CFU. g^{-1}), 45 DAS (24.5 $x10^6$ CFU. g^{-1}) and 60 DAS (17 $x10^6$ CFU. g^{-1}), Bt cotton rhizosphere

Table 4. Beneficial microbial population count from rhizosphere soil under *in-vitro* condition in different interval

Treatments	15 DAS		Mean	30 DAS		Mean	45 DAS		Mean	60 DAS		Mean
	non Bt cotton	Bt cotton		non Bt cotton	Bt cotton		non Bt cotton	Bt cotton		non Bt cotton	Bt cotton	
Control	0	0		0	0		0	0		0	0	
Azospirillum	2.0	1.0	1.5	30	50	38.9	20 (7.30)	24.5	22.3	12.5	17.0	14.7
brasilense	(6.30)	(6.00)	(6.15)	(7.48)	(7.70)	(7.59)		(7.39)	(7.35)	(7.10)	(7.23)	(7.17)
Pseudomonas	6.0	5.0	5.6	14.0	10.0	11.2	1.0	12.0	6.5	6.3	6.3	6.30
fluorescens	(6.78)	(6.72)	(6.75)	(7.17)	(7.00)	7.10)	(6.00)	(7.10)	(6.55)	(6.80)	(6.80)	(6.80)
Bacillus	10.0	8.0	9.0	50.0	44.0	46.0	45.0	47.0	46.0	10.0	13.0	11.0
megaterium	(7.0)	(6.9)	(6.95)	(7.7)	(7.65)	(7.67)	(7.66)	(7.68)	(7.67)	(7.0)	(7.12)	(7.06)
Trichoderma viride	1.0	1.5	1.2	3.0	2.0	2.4	2.0	3.1	2.5	2.2	2.2	2.1
	(3.00)	(3.18)	(3.09)	(3.48)	(3.30)	(3.39)	(3.30)	(3.50)	(3.40)	(3.35)	(3.30)	(3.33)
Mean	4.75	3.90		24.3	26.5		17.0	21.7		7.8	9.63	
	(5.77)	(5.70)		(6.46)	(6.41)		(6.07)	(6.40)		(6.06)	(6.11)	
	T	**C**	**Tx C**	**T**	**C**	**Tx C**	**T**	**C**	**Tx C**	**T**	**C**	**Tx C**
S.Ed	0.08	0.06	0.13	0.10	0.06	0.14	0.09	0.06	0.14	0.10	0.06	0.14
CD (p= 0.05)	0.18	NS	NS	0.21	NS	NS	0.20	NS	NS	0.20	NS	NS

-Microbial population is expressed in x 10^6 CFU. g^{-1}; T. viride x 10^3 CFU. g^{-1}
-Values in the paranthesis are log transformed values

registered higher population compared to non Bt cotton rhizosphere. *P. fluorescens* population was high (14 x 10^6 CFU. g^{-1}) in non Bt cotton on 30 DAS and in Bt cotton rhizosphere (12 x 10^6 CFU. g^{-1}) on 45 DAS. On comparison of Bt and non Bt cotton, population was more in Bt cotton at 45 DAS. *B. megaterium* also followed the same trend as *Pseudomonas* i.e., highest population was recorded at 30 DAS (50 x 10^6 CFU. g^{-1}) in non Bt cotton and 45 DAS (47 x 10^6 CFU. g^{-1}) in Bt cotton. The population was high in Bt cotton at 45 and 60 DAS. *T. viride* population recorded (3.1 x 10^6 CFU. g^{-1}) in Bt cotton and 3.0 x 10^6 CFU. g^{-1} in non Bt cotton on 45 and 30 DAS respectively (Table 4).

There was no significant effect of cultivar or cultivar x treatment interaction on the population of beneficial microbes at 15, 30, 45 and 60 DAS.

3.2 Molecular Analysis of the Functional Microbes

All the microbes were isolated at four intervals from pot culture (15, 30, 45 and 60 DAS) subjected for molecular analysis.

Total genomic DNA was isolated from the functional microbes recovered from Bt and non Bt cotton rhizosphere (three bacteria and one fungus) produced intact band on 0.8 per cent agarose gel confirmed that isolated DNA was pure and free from protein and RNA contamination. The quantity of DNA assessed by Spectrophotometry using Nanodrop, varied from 280 ng/µl to 3200 ng/µl of microbial culture. The OD_{260}/OD_{280} ranged between 1.83 and 2.03 indicating that there was no protein and RNA contamination.

To find out the variation if present within the isolates recovered from Bt and non Bt rhizosphere at different occasion of each microbe 16S rRNA was amplified from the all bacterial isolates, ITS region was amplified from the *T. viride* isolates and subjected to analysis of PCR-Restriction Fragment Length Polymorphism with *Hae*III and *Alu*I restriction enzymes.

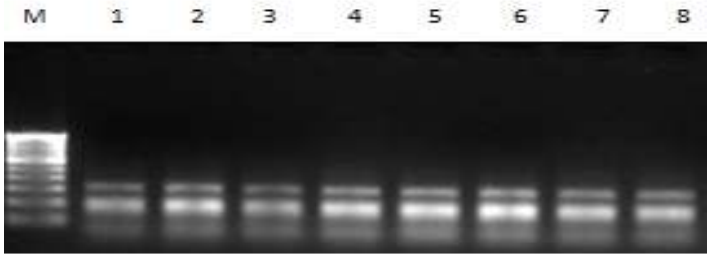
16S rRNA gene was amplified with all three beneficial bacterial isolates recovered from the Bt and non Bt cotton rhizosphere. All the isolates of *A. brasilense, P. fluorescens* and *B. megaterium,* recovered from Bt and non Bt cotton rhizosphere produced the amplicon around 1.46 kb, whereas *T. viride* recovered from Bt and non Bt cotton subjected for amplification of internal transcribed spacer (ITS) region with ITS1 and ITS4 primers produced amplicon size of 600 bp.

3.3 PCR-Restriction Fragment Length Polymorphism 16S rRNA and ITS Amplicons with *Hae*III and *Alu*I Restriction Enzymes

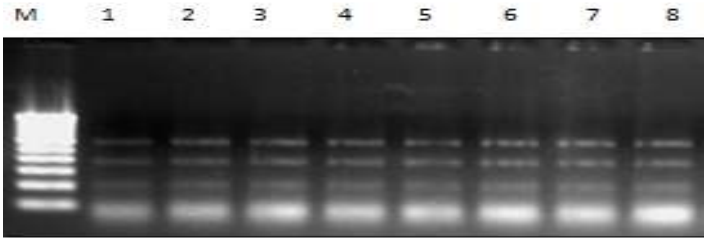
Eluted 16S rRNA products were used for restriction with two restriction endonuclease enzymes to find out the variation if any in the isolates recovered from Bt and non Bt cotton at different intervals (different exposure to the crystal protein). Restriction analysis of 16S rRNA from *A. brasilense* with *Hae*III and *Alu*I enzyme gave 350 bp, 200 bp and 80 bp fragments and 600 bp, 400 bp, 200 bp and 100 bp respectively (Fig. 1). All the 20 isolates (10 isolates from each from Bt and non Bt cotton) produced same restriction banding pattern. All the *P. fluorescens* isolates recovered from Bt and non Bt cotton produced bands with *Hae*III (450 bp, 300 bp, 200 bp, 150 bp and 100 bp) and 450 bp, 350 bp, 200 bp and 100 bp bands with the restriction enzyme *Alu*I (Fig. 2). Four bands in the size of 600 bp, 450 bp, 220bp and 120 bp with *Hae*III and three bands in the size of 600 bp, 250 bp and 100 bp with *Alu*I produced by the *B. megaterium* isolates recovered from Bt and non Bt cotton (Fig. 3). ITS region of *T. viride* produced the band in the size of 300 bp, 200 bp and 100 bp for *Hae*III and two band (450 bp and 150 bp size) to the *Alu*I enzyme (Fig. 4). Each banding pattern produced corresponds to a particular genotype. There was no difference observed in the banding pattern that is recovered at different interval with different exposure from the Bt cotton rhizosphere and respective non Bt cotton rhizosphere.

4. DISCUSSION

Present research work mainly focused on impact of Bt protein released from root exudates of Bt cotton on the selected functional microbes in green house condition. The samples extracted from rhizosphere soil of Bt cotton plants showed positive for the ELISA test. The result clearly indicated that Bt toxin was released from root exudates of Bt cotton. Similarly qualitative ELISA test was used to analyze the presence or absence of Cry1Ac and Cry2Ab protein in cotton by Downes et al. [12]. In another study, Rui et al. [13] concluded that no Bt toxin was found in the rhizosphere of non-Bt cotton SHIYUAN321, but Bt toxin was present in the rhizosphere of Bt cotton SGK321 and NuCOTN99B during the entire growth season.



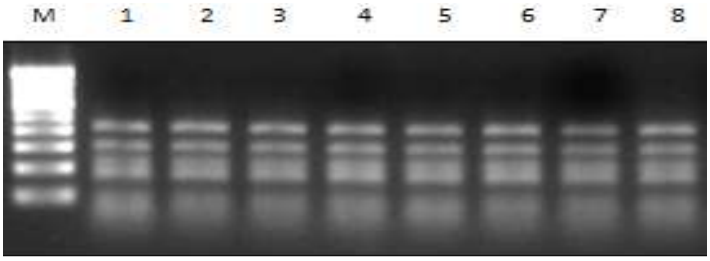
A. Banding pattern of *Hae*III (350 bp, 200 bp and 80 bp)



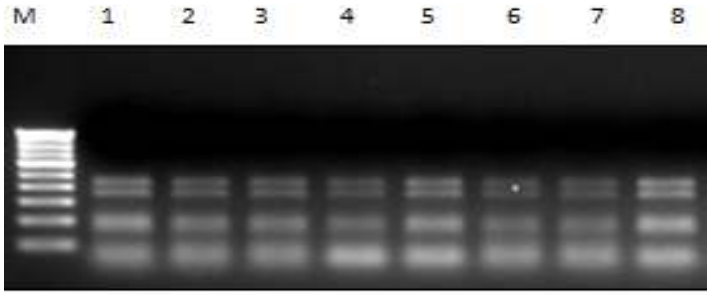
B. Banding pattern of *Alu*I (600 bp, 400 bp, 200 bp and 100 bp)

Fig. 1. PCR - RFLP of *A. brasilense* 16S rRNA

- *M-100 bp ladder; 1-4 non Bt cotton; 5-8 Bt cotton*



A. Banding pattern of *Hae*III (450 bp, 300 bp, 200bp,150bp and 100 bp)



B. Banding pattern of *Alu*I (450 bp, 350 bp, 200 bp and 100 bp)

Fig. 2. PCR - RFLP of *P. fluorescens* 16S rRNA

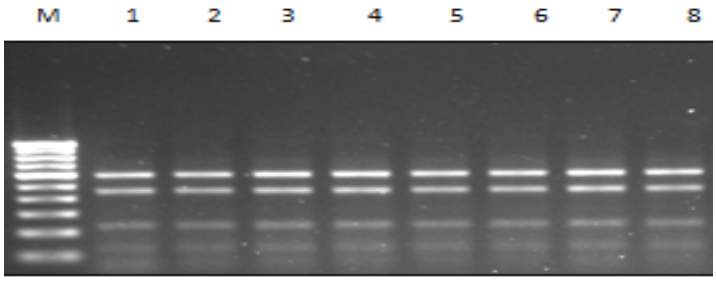
- *M-100 bp ladder; 1-4 non Bt cotton; 5-8 Bt cotton*

The rationale for the use of antibiotic resistance to mark bacteria in ecological studies is based on the unique advantages for recovery and enumeration, without interfering with backround

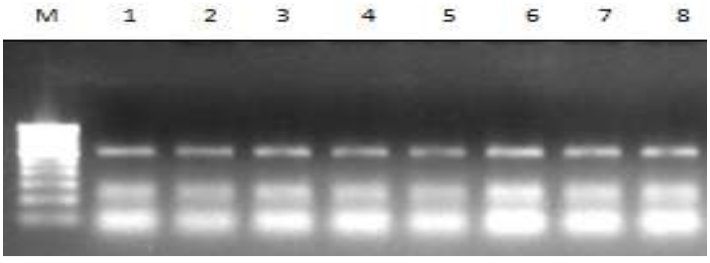
or native microbial population. In the present study based upon the resistance pattern of four selected microbes *A. brasilense, B. megaterium*, *P. fluorescens* and *T. viride* were able to differentiate these microbes form it's respective native population present in the rhizosphere soil Khan et al. [14] studied the antibiotic resistance profile of *B. megaterium* and *A. amazonense* to screen these isolates from its backround contaminants, and reported resistance level to various antibiotics.

Study conducted by Hu et al. [15] showed no consistent statistically significant differences between rhizosphere soil of Bt and non Bt cotton in the numbers of culturable nitrogen-fixing bacteria, bacteria that dissolve organic and inorganic phosphates and potassium-dissolving bacteria during the four sampling stages in the four fields. Some differences between Bt and non Bt cotton rhizosphere soil in the numbers of the various group of functional bacteria were observed but these differences were not consistent from one sampling stage to the next.

In the present study, the selected beneficial microbial population was evaluated in rhizosphere soil of transgenic Bt and non Bt cotton. Beneficial microbial population was high in 45 and 60 DAS of Bt cotton rhizosphere when compared with non Bt cotton rhizosphere. But the difference is statistically not showed any significance. These results are in line with the findings of Icoz et al. [16]. Similarly, Saxena and Stotzky, [5] also did not find any significant differences in the numbers of culturable bacteria, actinomycetes, fungi, protozoa, and nematodes in the rhizosphere of transgenic Bt and non-transgenic maize. In addition, Blackwood and Buyer [17] assessed the effects of two lines of transgenic corn expressing different Cry endotoxins on the soil microbial community structure in three soil types in a growth chamber experiment; the bacterial community structure was less affected by transgenic Bt corn than by the soil type. Swilla et al. [18] suggested that Cultivation of Bt cotton line 06Z604D expressing both Cry1Ac and Cry2Ab2 protein did not result in significant change in the overall numbers of culturable bacterial and fungal populations; and transgenic line 06Z604D had no clear effect on the number of culturable bacterial and fungal populations in the rhizosphere within one growing season. Overall the soils exposed to Bt did not show significant variation in the bacterial and fungal populations



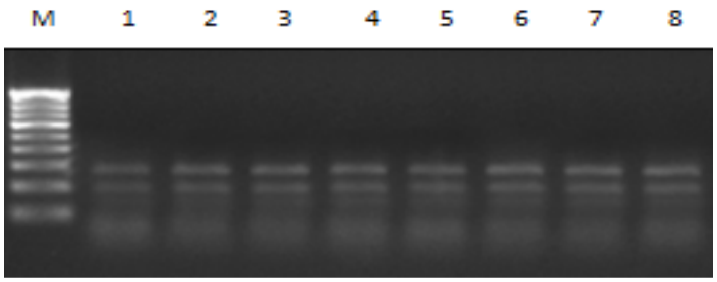
A. Banding pattern of *Hae*III (600 bp, 450 bp, 220 bp and 120 bp)



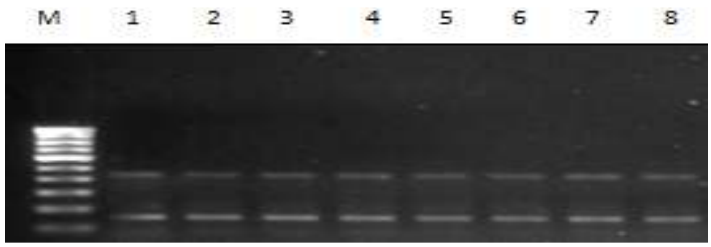
B. Banding pattern of *Alu*I (600 bp, 250 bp and 100 bp)

Fig. 3. PCR - RFLP of *B. megaterium* 16S rRNA

- *M-100 bp ladder; 1-4 non Bt cotton; 5-8 Bt cotton*



A. Banding pattern of *Hae*III (300 bp, 200bp and 100 bp)



B. Banding pattern of *Alu*I (450 bp and 150 bp)

Fig. 4. PCR – RFLP of *T. viride* ITS region

- *M-100 bp ladder; 1-4 non Bt cotton; 5-8 Bt cotton*

Brusetti et al. [19] detected no differences in the rhizosphere bacterial communities between transgenic Bt 176 maize and its non-transgenic counterpart. Other authors, however, have reported minor to significant effects of Cry proteins and transgenic Bt crops on microbial community structure in soil. Petras and Casida, [20] observed slight increases in populations of bacteria, actinomycetes, fungi, and nematodes after the addition of *B. thuringiensis* subsp. *kurstaki* to the soil; Petras and Casida, [20] inferred that the crystal proteins were used as a substrate. A significant but transient increase in the populations of culturable bacteria and fungi was observed in soil with leaves of Bt cotton *(Gossypium hirsutum L.)* expressing the Cry1Ac protein [21]. Velmourougane and Sahu [22] study on cultivation of Bt cotton revealed that cultivation of transgenic Bt cotton expressing cry1Ac gene had no adverse effects on soil biological activities such as soil respiration, urease, dehydrogenase, fluorescein diacetate hydrolysis, microbial biomass carbon, culturable microbial population and microbial diversity indices. Based on the overall observations, growing Bt cotton was found to have a positive impact on soil biological activities and may not pose ecological or environmental risk.

Rui et al. [13] found higher numbers of functional bacteria in the rhizosphere soil of Shiyuan 321 (non-Bt cotton) than NuCOTN99B (Bt cotton); after adding pure Bt toxin to soil, also indicated that Bt toxin may not directly affect the numbers of functional bacteria in the rhizosphere.

In the present investigation temporal population variation and functional activity at a different time interval observed but the observed variation was transient. this result was confirmed by swilla et al. [18], they found that The effect of transgenic plants on soil populations of non-target bacteria and fungi could be either transient or do not have any effect at all. Temporal variations in microbial population were observed between Bt and non-Bt cotton were attributable to differences in genetic makeup of the cotton hybrids rather than gene expression [22].

So molecular study was carried out with all the isolates recovered from the different intervals from Bt and non Bt cotton rhizosphere to check whether this variation is because of difference occurred in the gene level.

16S rRNA, ITS, gene were amplified with gene specific primers and the expected amplicons were obtained for the each gene with respective organism. Similar research, amplification 16S rRNA, ITS, gene have been reported by many researchers [23,24]. In the present investigation restriction analysis of 16S rRNA from all the eight

isolates (four isolates from each from Bt and non Bt cotton) *A. brasilense, P. fluorescens, B. megaterium* and *T. viride* produced same restriction banding pattern. Each banding pattern produced corresponds to a particular genotype. There were no difference observed in the banding pattern among the isolates recovered from the Bt and non Bt cotton rhizosphere of each beneficial microbe. Similar type of research was conducted by Nordeen et al. [25] to determine the effect of cecropin B produced from transgenic potato on non target *Bacillus* communities in the rhizosphere, who found rhizospheres of transgenic and parental potatoes had comparable *Bacillus* community structures and diversities at the tuber production stage.

5. CONCLUSION

Differences in the functional bacteria population between rhizosphere soil of Bt and non-Bt cotton in the pot culture under green house either transient or absent. There was no population reduction in Bt cotton when compared to its counterpart. So it clearly shows in green house condition (short term) Bt toxin doesn't have any effect on *Azospirillum brasilense*, *P. fluorescens*, *Bacillus megaterium* and *Trichoderma viride* population. Since this is minimal study still we need various composite experiments (cultural and molecular level) and long term analysis to prove the safety of the transgenics against the functional microbes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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