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BACTERIAL AND FUNGAL DIVERSITY IN RHIZOSPHERE SOILS OF Bt AND NON-Bt COTTON IN NATURAL SYSTEMS

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Abstract

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Bacteria are the most diverse living beings on earth and only a fraction of them have been identified. This study aims at the analysis of the diversity of bacteria and fungi in rhizosphere soils of Bt and non-Bt cotton fields, which were collected from four different locations of Mahabubnagar District, Andhra Pradesh, India. Bacterial and fungal genera identification was done based on 16S rRNA and 18S rRNA gene sequences respectively. The results regarding bacterial and fungal diversity revealed 6 and 7 predominant genera resp. in Bt and non-Bt cotton species. The most abundant representatives belong to the *Azospirillum*, *Bacillus* and *Pseudomonas* in bacteria and and *Fusarium, Aspegillus, Rhizopus and Alternarica* in fungi. Results revealed that non-Bt cotton plant growth is more than Bt cotton plant growth and rhizosphere soil sample of non-Bt cotton has shown increased number of bacterial and fungal populations indicating adverse effects on soil micro flora.

Key words: cotton, rhizosphere, bacteria, fungi, diversity

Introduction

In India 9 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 (James, 2008). 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 (Saxena and Stotzky, 2001; Mina et al., 2008; Liu, 2009). Bt toxin released in soil get adsorbed or bound on clay particles, humic components, or organic mineral complexes and then be protected against degradation by soil microorganisms (Tapp et al., 1995). Although Bt toxin is found naturally in many soils, continuous growing of Bt crops on same location enhances its existing levels to a certain concentration that might affect the composition and activity of soil microbial communities (Donegan and Seidler, 1999; Stotzky, 2004; Wei et al., 2006, Griffiths et al., 2007; Rui et al., 2005) and the soil biochemical properties (Rui et al., 2005; Fang et al., 2007; Sun et al., 2007; Sarkar et al., 2009).

Several experiments were conducted to assess risk of Bt cotton on flora and fauna in diverse agroecosystems (Zhang et al., 2000; Li et al., 2002; Liu et al., 2002; Men et al., 2003; Bai et al., 2003). Some studies indicate that Bt cotton has no negative effects on soil flora and fauna and may even have beneficial effects (Saxena and Stotzky, 2001; Sarkar et al., 2009), while some have reported adverse effects (Cui and Xia, 2000; Tan et al., 2002).

This research aimed the evaluation of the bacterial and fungal diversity in rhizosphere soils of BT and non-BT cotton samples collected from four cotton fields in Mahabubnagar District, through enumeration and identification of bacterial and fungal species present in rhizosphere soils of cotton fields.

Materials and Methods

This study was carried out in the Department of Microbiology, Palamuru University, Mahabubnagar, Andhra Pradesh, India.

Soil sampling

Soil samples were collected from the rhizosphere of Bt (Mahyco, Marvel, Diana and Rashi) and non-Bt (Kaveri, Su-

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per nova, Super seeds and N-32) cotton varieties grown in four areas of Mahabubnagar District viz. Achempet, Balnagar, Nagarkurnool and Kalwakurthy after 90 days sowing. Area of 1ft around the plant was marked and represented as the rhizosphere soil. Each rhizosphere soil sample comprised of 5 core samples drawn from 5-6 inches deep and 3 inch wide bore made around the rhizophere of 5 randomly selected Bt plant. The soils was drawn by the same procedure from the rhizosphere of non-Bt plants too. The soil from the two treatments were mixed thoroughly in separated containers. From this 1 g soil was drawn as representative rhizosphere sample.

Growth parameters analysis: BT and non-Bt cotton plants

Cotton plants of respective rhizosphere soils were taken for the measurement of growth parameters. The observations on different growth parameters were recorded for 90 days old plants. The length of the shoot was adjusted by taking the physical count from color region to apical bud. The length of the root was adjusted by taking the physical count of the length of root from color region to the tip of the taproot. The fresh root & shoot samples were measured physically on the top loading balance & resulting weight were recorded as shoot & root fresh weight in grams. The dry matter accumulation by root & shoot was recorded by subjecting the root and shoot to oven drying at 60ºC.

Determination of total bacterial and fungal population

To determine the total bacterial and fungal populations, rhizosphere soils were homogenized in a pestle to break the clods. Samples were prepared in sterile distilled water by suspending 1 gm soil in 100 ml water and shaking the sample vigorously for 20 min on an orbital shaker at 250 rpm. The primary soil suspensions were serially diluted further and 10^{-4} dilutions were plated on nutrient agar (NA), Pikovaskys agar, titan media, Jensens media, Soyabean tripticase agar, PC (Phenonthroline Columbia) media and Rose-Bengal supplemented potato dextrose agar (PDA) media in Petri plates to determine the populations of bacterial and fungal micro flora respectively. For fungal media 40 mg/l gentamycin and 100 mg/l, Chloramphenicol was added to inhibit bacterial growth. The plates were incubated at 27-30ºC for 3 and 7 days for bacteria and fungi respectively and observed for the appearance of colonies. The population count of the organisms was recorded after the stipulated incubation periods.

Genomic DNA Extraction

The genomic DNA from the bacterial cells was obtained using a modification of the method described by Sambrook et al. (1989). The bacterial cells from pure culture were harvested by centrifugation (12 000 rpm) for 2 min, and the cell pellets mixed with 600µl of lysis buffer (10 mm tris –HCl, 1mM EDTA [pH 7.5], 0.5% SDS 100 g/ml proteinase K) were incubated at 37˚C for 1h after the addition of 100 µl 5 M NaCl, and 80 µl CTAB NaCl. Samples were incubated at 65˚C for 10 min. The samples were cooled to room temperature, followed by extraction of the aqueous phase with an equal volume of chloroform: isoamyl alcohol [24:11, v/v] and then with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1, v/v) which was centrifuged at 12 000 rpm and 4˚C for 10 min. Isopropanol (0.6x) was mixed with the aqueous phase, and centrifuged at 12 000rpm and 4˚C for 10 min. The DNA pellets were dried under vacuum, and then dissolved in TS Buffer (10mM Tris-HCl, and 1Mm EDTA [pH 7.5]).

PCR Analysis

The small subunit rRNA gene of each sample's culture DNA was amplified using 16S rRNA Universal primers. The PCR amplification reaction mixture of 50 µl contained 4 µl bacterial DNA (nearly 200 ng), 1µl Taq-DNA polymerase, 5 µl of Taq buffer, 5 µl of 2 mM dNTP mix, 5 µl of forward primer (10 pM/µl) and 5 µl of reverse primer (10 pM/µl). Amplification was carried out in a Bio-Rad thermo cycler for 30 cycles. In each cycle denaturation was done at 94ºC for 20 s, annealing at 48ºC for 20 s and extension was done at 72ºC for 40 s and a final extension was carried out for 5min at 72ºC at the end of all 30 cycles. The amplified DNA fragment of approximately 1542 bp was separated on a 1% agarose gel and purified by Quiagen spin columns (Stirling, 2003).

The desired DNA band from the agarose gel was cut weighed and then transferred to a sterile microfuge tube and QE buffer thrice the volume of weighed excised band was added. Place it on a thermomixer at 65ºC for 10min and the contents were then transferred to a Quiagen column and spun at 8000 xg for 2 min. Then it was washed with 750 µl of PE buffer and eluted with small quantity (30-40 µl) of sterile water. The purified PCR product was then used for sequencing.

16S rRNA gene Sequencing

The purified 1542bp PCR product was sequenced using universal primers. The resultant almost complete sequence of the 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search (Altschul et al., 1990) and Ez Taxon (Chun et al., 2007) to identify the nearest taxa. The entire related 16S rRNA gene sequences were downloaded from the database (http://www.nbi.nlm.nih-gov) aligned using the clustal – program (Thompson et al., 1997).

18S rRNA gene Sequencing

Whenever macro- and micro morphology failed to show unambiguous results, PCR of the gene coding for the ribosom-

al internal transcribed spacers (ITS) with the enclosed 5.8S ribosomal DNA and subsequent sequencing was performed. DNA from fungi was isolated. Thereafter, the ITS region was amplified by PCR using the primer set. Sequencing of the amplified ITS region was accomplished according to the Sanger-Coulson method (or chain termination method using single-stranded DNA) with subsequent analysis of the sequenced products using the Genetic analyzer ABI PRISM 3130. The ITS sequences were then compared with entries in genomic databanks using the Internet free-ware from European Bioinformatics Institute(EMBL) found under http://www.ebi.ac.uk/fasta33/nucleotide.html to identify the specific fungi

Results and Discussion

Results of plant parameters of Bt and non-Bt cotton are presented in Figure 1a and 1b. From the results, it is clear that plant parameters of non-Bt cotton are higher than Bt cotton. Total number of bacterial and fungal colonies was counted on respective media. The results of the bacterial study are presented in Table 1a and 1b. Populations of rhizosphere bacteria of non-Bt cotton are higher than Bt cotton. The types of colonies, which grew from Bt cotton and non-Bt cotton, were similar. The bacterial strains viz., *Rhizobium* sp., *Azospirillum* sp., *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Actinomycetes* sp., etc were predominant in the rhizosphere of two plant types but their number is high in non-Bt cotton rhizosphere soil.

The total fungal populations in the Rhizosphere of Bt and non-Bt cotton samples are presented in Table 2a and 2b. Species of *Fusarium* were the predominant fungi in rhizosphere of Bt and the non-Bt cotton. Besides these, species of *Aspergillus, Alternaria, Rhizopus, Rhizoctonia, Trichoderma* and *Penicillum* were some of the other common fungi found in the rhizosphere of both the types of plants but their number is highest in non-Bt cotton rhizosphere soil when compared with Bt cotton.

There are several reports that genetically modified plants changed the behavior of microbe rhizosphere. Donegan et al. (1996) studied that the environmental release of GM plants may be accompanied by ecological effect including changes in plant associated microflora. Donegan et al. (1999) in a case of transgenic cotton containing Bt-toxin placed into soil in a laboratory study to observe decomposition of plant residue and found differences in carbon content between the decomposing parental and Bt cotton litter due to soil microbial populations. DiGiovanni et al. (1999) found in case of *Medicago sativa* transgenic and normal varieties difference in soil microorganisms. Shen et al. (2006) studied microbial activities affected due to transgenic Bt cotton (Sukang-103) and its non-Bt cotton counterpart (Sumian-12) were investigated to evaluate potential risk of transgene on soil ecosystem. Savka and Farrand (1997) very recently demonstrated that in a legume plant Lotus corniculatus opine producing GM plant altered its microbial environment in rhizosphere.

It is clearly evident from the results that non-Bt cotton plant growth is more than Bt cotton plant growth and rhizosphere soil sample of non-Bt cotton has shown increased number of bacterial and fungal populations. It is apparent from the results of the microbial population analyses that Bt plants caused changes in the cultural soil bacteria and fungi. Results showed that Bt cotton has adverse effect on soil micro flora and were not safe for rhizosphere organisms.

Conclusion

A significant variation in Rhizosphere soil microbial count (both bacterial and fungal) of Bt mad non-Bt clearly demonstrated the transgenic effect of Bt cotton on soil flora. The microbial abundance in the non Bt soil has proven the non-toxication,

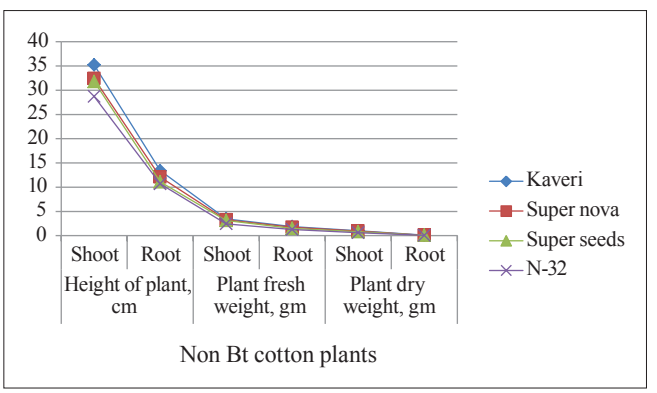

Fig. 1a. Plant parameters of 90 days old non-Bt cotton collected from four regions of Mahabubnagar District

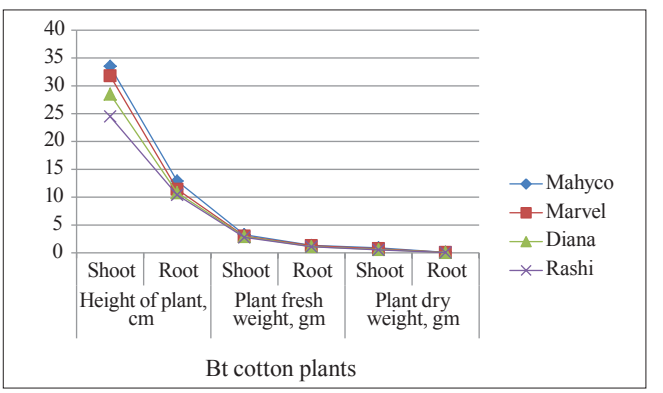

Fig. 1b. Plant parameters of 90 days old Bt cotton collected from four regions of Mahabubnagar District

Table 1 (a)
Bacterial isolates identified in rhizosphere soils of non-BT cotton from four regions in Mahabubnagar District. (Achampet, Balnagar, Nagarkurnool and Kalwakurthy)

Bacteria	Non Bt Cotton				Total number of isolates	Frequency of isolates, %
	Kaveri	Super nova	Super seeds	N-32		
Rhizobium	26	-	26	20	72	14.06
Azospirillum Sp	20	28	22	25	95	18.55
Azotobacter	22	30	-	28	80	15.62
Actinomycetes	-	22	27	29	78	15.23
Bacillus Sp	30	27	22	16	95	18.55
Pseudomonas fluorescence	18	24	21	29	92	17.96
Total					512	100

Table 1 (b)
Bacterial isolates identified in rhizosphere soils of BT cotton from four regions in Mahabubnagar District. (Achampet, Balnagar, Nagarkurnool and Kalwakurthy)

Bacteria	Bt Cotton				Total number of isolates	Frequency of isolates, %
	Mahyco	Marvel	Diana	Rashi		
Rhizobium	18	-	18	19	55	16.17
Azospirillum Sp	15	12	18	18	63	18.57
Azotobacter	14	17	20	-	51	15
Actinomycetes	17	19	-	16	52	15.29
Bacillus Sp	13	11	16	18	58	17.05
Pseudomonas fluorescence	18	15	14	14	61	17.94
Total					340	100

Table 2 (a)
Fungal isolates identified in rhizosphere soils of non BT cotton from four regions in Mahabubnagar District. (Achampet, Balnagar, Nagarkurnool and Kalwakurthy)

Fungi	Non Bt Cotton				Total number of isolates	Frequency of isolates, %
	Kaveri	Super nova	Super seeds	N-32		
Aspergillus Sp	22	18	22	16	78	15.11
Penicillium	23	-	25	17	65	12.59
Rhizopus	14	20	23	15	72	13.95
Fusarium	19	26	25	21	91	17.63
Trichoderma	25	24	-	17	66	12.79
Alternaria	20	19	16	18	73	14.14
Rhizoctonia	18	15	18	20	71	13.75
Total					516	100

Table 2 (b)
Fungal isolates identified in rhizosphere soils of BT cotton from four regions in Mahabubnagar District. (Achampet, Balnagar, Nagarkurnool and Kalwakurthy)

Fungi	Bt Cotton				Total number of isolates	Frequency of isolates, %
	Mahyco	Marvel	Diana	Rashi		
Aspergillus Sp	10	11	12	6	39	12.95
Penicillium	10	12	16	-	38	12.62
Rhizopus	13	14	9	11	47	15.61
Fusarium	15	13	16	13	57	18.93
Trichoderma	12	17	8	9	46	15.28
Alternaria	10	10	-	8	28	9.3
Rhizoctonia	15	10	10	11	46	15.28
Total					301	100

while the toxic effect of *Cry1AC* gene has depleted the microbial abundance. Therefore, Bt may not be safe for the non-targeted microbes including Rhizosphere bacteria and fungi.

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