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Effects of root exudates of transgenic cotton (Bt cotton) plants on selected plant growth promoting microbes

Sivaji Mathivanan and Sridar Raghupathi

Abstract
The present study was undertaken to evaluate the effects of root exudates of transgenic cotton (Bt cotton) plants on selected plant growth promoting microbes. Root exudates were collected from the Rhizosphere soil of Bt and non Bt cotton (RCH BG II and RCH respectively) at 30 days after sowing (DAS) and 60 days after sowing (DAS) under *in vitro* condition and incorporated in to the respective broth of selected beneficial microbes (*Azospirillum brasilense*, *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma viride*). Presence of Cry 1 Ac and Cry 2 Ab (endo-toxins) in Bt cotton root exudates was confirmed with qualitative Desi-Gen ELISA Kit assay. The population count results revealed that no population variation on any of the microbes which were tested in both the root exudates taken from 30 and 60 DAS of the Bt and non Bt cotton. Among four microbes *A. brasilense* was found in high numbers and the least was *T. viride*. At 30 DAS *A. brasilense* and *P. fluorescens* were higher in Bt cotton where as *B. megaterium* and *T. viride* were found to be high in non Bt cotton. On 60 DAS *A. brasilense* and *T. viride* was more in Bt cotton, *P. fluorescens* was more in non Bt cotton. The population of *B. megaterium* was on par in both Bt and non Bt cotton. However there were no significant differences observed between cultivar in both the stages (30 and 60 DAS) on functional microbes.

Keywords: Bt cotton, Elisa test, Cry protein, Root exudates, beneficial microbes

1. Introduction
The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root [1]. The rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighbouring plant species for space, water and mineral nutrients, and with soil borne microorganisms, including bacteria, fungi and insects feeding on an abundant source of organic material [2]. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root and by microorganisms feeding on the compounds. The exudates of plants are chemically complex in nature containing carbohydrates, lipids, proteins, organic acids and some other secondary metabolites [2]. As plant roots grow through soil they supply food for the microorganisms by producing root exudates. The food supply influence microbiological activity in the rhizosphere which is greater than in soil away from plant roots [3, 4].
The rhizosphere microflora colonization depends on characteristics of soil such as soil texture, soil pH, temperature, plant species and even plant genotype and root exudates of the plants [5, 6]. GM plants have the potential to significantly change the microbial dynamics and essential ecosystem functions such as nutrient mineralization, disease incidence, carbon turnover and plant growth through the products of introduced genes or modified rhizosphere chemistry or altered crop residue quality [7] also the exudates of Bt cotton plants are found to be rich in endotoxin *i.e.,* Cry protein [8].
The compounds secreted by plant roots serve important roles as chemical attractants and repellents in the rhizosphere, the narrow zone of soil immediately surrounding the root system [9, 10]. The chemicals secreted into the soil by roots are broadly referred to as root exudates [11]. Through the exudation of a wide variety of compounds, roots may regulate the soil microbial community in their immediate vicinity, cope with herbivores, encourage beneficial symbioses, change the chemical and physical properties of the soil and inhibit the growth of competing plant species [11]. The ability to secrete a vast array of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with nearly 5 % to

21 % of all photosynthetically fixed carbon being transferred to the rhizosphere through root exudates [12]. Changes in biochemical properties of rhizosphere soil can reflect the influence of plant on soil biochemical properties [13]. Root exudates play an important role in microflora selection and selectively influence microbial growth. In fact qualitative and quantitative differences in root exudation can strongly affect the structural and functional diversity of the rhizosphere population [13].

Plants and microorganisms interact with each other and co evaluate together. It is widely acknowledged that root exudates govern which organisms should reside in the rhizosphere. Therefore, any change in the quality and quantity of root exudates could potentially modify the biodiversity of soil microbiota and may cause changes in both harmful and beneficial microorganisms. So present study mainly focussed on to study the effects of transgenic cotton root exudates on selected plant growth promoting microbial population (Beneficial microbes).

2. Materials and Methods

2.1 Collection of cotton seed and beneficial microbial cultures

Bt and non Bt cotton seeds used in this study were obtained from Rasi Seeds Pvt. Ltd., Attur. The cultures of *Azospirillum brasilense*, *Bacillus megaterium* (biofertilizers), *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.

2.2 ELISA test

Bt cotton (RCH BG II) and non Bt cotton (RCH cultivar) root exudates was collected from 30 days old plants. Presence of Cry 1Ac and Cry 2Ab toxin in root exudates was checked by using DesiGen qualitative ELISA kit by following manufacturer protocol [14].

2.3 Collection of root exudates form Bt and non Bt cotton

Root exudates study was conducted by following the method described by Brusetti *et al*. [15] with slight modification. Cotton seeds were imbibed for 1 h in distilled H_2O and then surface sterilized for 20 min in 2 % sodium hypochlorite.

The seeds were then placed on glass wool (solid platform) in the growth container containing 50 ml of Hoagland solution and kept in the green house condition (Fig.1). Hoagland solution composition was given in Table 1. After 30 and 60 days growth the root exudates from Bt and non Bt cotton were collected, filter sterilized through a 0.22 µm filter and stored at -80 ºC. Then 40 ml of root exudates collected from Bt and non Bt cotton was mixed with 60 ml of malic acid, Luria Bertani (LB), King's B (KB) and Potato Dextrose broth separately with inoculated the respective microorganism and incubated for 15 days in four replications. After 15 days incubation, 1 ml culture was taken from each replication and mixes it together. Then the mixed sample was serially diluted up to 10^{-8} times from this 100 µl cultures were taken and spread on respective agar media *i.e., A. brasilense, B. megaterium, P. fluorescens* and *T. viride* on malic acid, LB, King's B and Potato Dextrose Agar media respectively (four replication each). The colony count of each microorganism was taken from the average of four plates and expressed as CFU ml^{-1}.

2.4 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.

3. Results and Discussion

3.1 ELISA test

The root exudates samples of Bt cotton plants showed positive for the ELISA test by turning yellow colour in the ELISA plate wells whereas non Bt cotton root exudates 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 Bt protein was released from root exudates of Bt cotton. Similar approach was adopted to analyze the presence or absence of Cry1Ac and Cry2Ab protein in cotton [16, 17].

3.2 Root exudates study

Root exudates collected from Bt and non Bt cotton was incorporated with the respective culture broth and inoculated with *A. brasilense, B. megaterium, P. fluorescens* and *T. viride* and observation was taken after ten days incubation by following serial dilution method. The appearance of the microbial colonies on the surface of the agar plates were shown in Fig. 2. The results showed no population variation on any of the microbes which were tested in both the root exudates taken from 30 and 60 DAS of the Bt and non Bt cotton. Among four microbes *Azospirillum* was found in high numbers and the least was *T. viride*. At 30 DAS *A. brasilense* (69.2×10^9 CFU. ml^{-1}) and *P. fluorescens* (4×10^9 CFU. ml^{-1}) were higher in Bt cotton whereas *B. megaterium* (6.9×10^9 CFU. ml^{-1} and *T. viride* (8.9×10^8 CFU. ml^{-1}) were found to be high in non Bt cotton. On 60 DAS *A. brasilense* (89×10^9 CFU. ml^{-1}) and *T. viride* 6.9×10^8 CFU. ml^{-1} was more in Bt cotton, *P. fluorescens* was more 2.8×10^9 CFU. ml^{-1} in non Bt cotton. The population of *B. megaterium* was on par (4.9×10^9 CFU. ml^{-1}) in both Bt and non Bt cotton. However there were no significant differences observed between cultivar and interaction between cultivar and treatment in both the stages (30 and 60 DAS) on functional microbes (Table 2).

It is widely acknowledged that root exudates govern which organisms reside in the rhizosphere [18, 19]. Therefore, any change in the quality and quantity of root exudates could potentially modify the composition (biodiversity) and activity of the soil microbiota and may cause changes in both deleterious and beneficial microorganisms. For example, a decrease in specific microbial populations could lead to a decrease in decomposition processes, alter the level and composition of soil organic matter and have secondary effects on the survival of plant pathogens [20]. In the present investigation the root exudates of Bt cotton at 30 and 60 DAS had no deleterious effect on *Azospirillum brasilense, Pseudomonas fluorescens, Bacillus megaterium* and *Trichoderma viride.* Some minor changes were observed in terms of microbial population with incubation with root exudates between Bt cotton (RCH2 BG II and its counterpart non Bt cotton (RCH2) but the difference was statistically not significant.

However, Gupta and Watson [7] reported that the microbiota

(*e.g.,* bacteria, actinomycetes, fungi) associated with residues of Bt cotton were significantly different from those associated with residues of herbicide tolerant cotton (Roundup Ready), but more extensive colonization by fungi (spores and hyphae) was observed in residues of Bt cotton than in residues of non Bt cotton. Turrini *et al.* [21] found that root exudates of Bt maize (event 176) significantly reduced presymbiotic hyphal growth of the arbuscular mycorrhizal fungus, *Glomus mosseae* compared with root exudates of another Bt maize hybrid (event Bt11) and non Bt maize.

It was suggested that the exudates of Bt maize differed from those of non Bt maize in several ways and not only in the content of the Cry protein [14]. Various studies, using culture independent methods showed some minor or no Bt specific effects on soil microorganisms and plant age and type appeared to be the major factors affecting bacterial diversity [22, 23]. Donegan *et al*. [24] found that the endotoxin from *B. thuringiensis* sub sp. *kurstaki* both purified and produced in transgenic plants, did not have a direct effect on soil microorganisms and that the effects observed, which were related to the plant varieties used, may have been caused by unexpected changes in plant characteristics that resulted from genetic manipulation or tissue culturing of the engineered plants.

Table 1: Composition of Hoagland solution

Component	Stock Solution (g l^{-1})	ml Stock Solution/1000 ml
Macronutrients		
2M KNO_3	202	2.5
1M $Ca(NO_3)_2.4H_2O$	236	2.5
Iron	15	1.5
2M $MgSO_4.7H_2O$	493	1
1M NH_4NO_3	80	1
Micronutrients		
H_3BO_3	2.86	1
$MnCl_2.4H_2O$	1.81	1
$ZnSO_4.7H_2O$	0.22	1
$CuSO_4.5H_2O$	0.051	1
$H_3MoO_4.H_2O$ or	0.09	1
$Na_2MoO_4.2H_2O$	0.12	1
Phosphate		
1M KH_2PO_4 (pH to 6.0)	136	0.5

Table 2: Effect of Bt protein from root exudates of Bt and non Bt cotton on population of functional microbes

Treatments	30 days		Mean	60 days		Mean
	non Bt cotton	Bt cotton		non Bt cotton	Bt cotton	
Azospirillum brasilense	59.0 (10.77)	69.2 (10.84)	64.1 (10.81)	79.4 (10.90)	89.1 (10.95)	85.0 (10.93)
Pseudomonas fluorescens	1.2 (9.30)	4.0 (9.6)	2.6 (9.45)	2.8 (9.45)	2.0 (9.30)	2.4 (9.38)
Bacillus megaterium	6.9 (9.84)	5.9 (9.77)	6.4 (9.81)	4.9 (9.69)	4.9 (9.69)	4.9 (9.69)
Trichoderma viride	0.89 (8.95)	0.69 (8.84)	0.79 (8.90)	0.59 (8.77)	0.69 (8.84)	0.64 (8.81)
Mean	16.7 (9.72)	19.9 (9.76)		21.9 (9.70)	24.2 (9.70)	
	T	**C**	**TxC**	**T**	**C**	**TxC**
S.Ed	0.20	0.13	0.29	0.20	0.13	0.29
CD (p= 0.05)	0.42	NS	NS	0.42	NS	NS

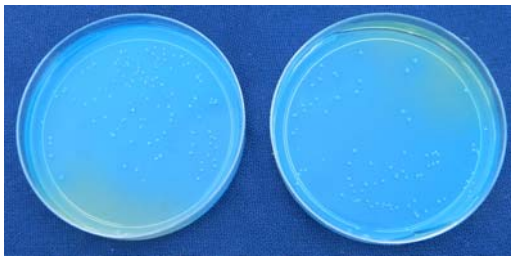
-Microbial population is expressed in x 10^9 CFU. ml^{-1}

-Values in the parenthesis are log transformed values

Cotton plants in glass tubes

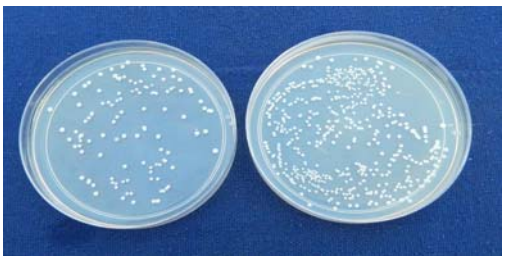
Growth of Cotton roots in Hoagland solution

Fig 1: Root exudates study



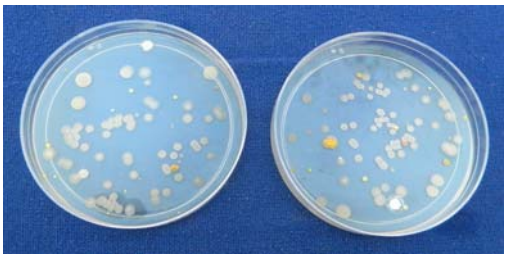
non Bt cotton Bt cotton

Azospirillum brasilense



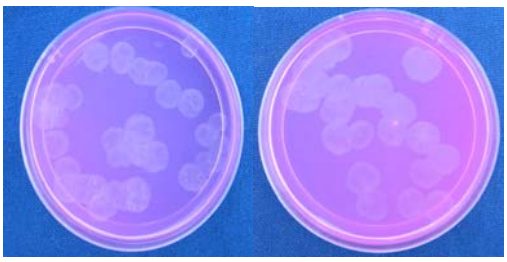
non Bt cotton Bt cotton

Pseudomonas fluorescens



non Bt cotton Bt cotton

Bacillus. megaterium



non Bt cotton Bt cotton

Trichoderma viride

Fig 2: Microbial growth of non Bt and Bt cotton on agar plates

4. Conclusion

The exudates of Bt cotton plants are found to be rich in endotoxin i.e., Cry protein. Since the cry toxin is bacterial origin (*B.thuringiensis*) it may affect the other beneficial microorganism which resides Bt cotton rhizosphere. Even though the Bt cotton root exudates contain cry protein there was no population reduction in Bt cotton when compared to its counterpart. So conclusion of this short term study is Bt toxin doesn't have any effect on *Azospirillum brasilense*, *P. fluorescens*, *Bacillus megaterium* and *Trichoderma viride* population.

5. Acknowledgement

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