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RESEARCH ARTICLE

Persistence and Enzymatic Activities Assessments of Larvicidal Crystal Proteins from *Bacillus thuringiensis* Bollgard II Cotton in Bioinoculant Rhizosphere Soils

King Solomon Ebenezer and Rajesh Kannan Velu*

Rhizosphere Biology Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli- 620024, TamilNadu, India.

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Corresponding Author

King Solomon Ebenezer

Abstract

The present research aimed to investigate the ecological risk of any transgenes release of *Bacillus thuringiensis* (*Bt*) cotton (Bollgard II) and non-*Bt* cotton (SVPR2) in the bioinoculants (Azospirllum and phosphate solublizing bacteria) treated rhizosphere soils. Rhizosphere soil enzymes like dehydrogenase, phosphatase, urease, invertase, protease levels and insecticidal crystal proteins were assessed and were explored by using ELISA. Few significant differences in enzyme levels in Azospirillum treated rhizosphere soil compared to phosphate solublizing bacteria inoculant and ELISA was indicated that *Bt* cotton bollgard II expressed Cry2Ab protein on extremely very negligible amount in soil and Cry1Ac persistence was absence. That there are no significant effects of *Bt* cotton on both bioinoculants treated rhizosphere soil. More importantly, this study demonstrates a comprehensive system for assessing the impacts of genetically modified *Bt* cotton even in bioinoculant treated rhizosphere soils. This is the first report on *Bt* cotton in bioinoculants soils.

INTRODUCTION

Cotton is a most important cash crop and also major basic with raw materials for textile industries. This cotton is most defenseless to some insects like Lepidopteron, so the cotton breeders are incessantly looking forward to control them from insects. Recently, biotechnology researchers have been produced the second generation *Bt* cotton (Bollgard II), which is insect resistant crop could be contains Cry1Ac and Cry2Ab insecticidal crystal proteins from *Bacillus thuringiensis* bacterium in 2003/2004 season (Tabashnik et al., 1996). These modified *cry* genes are inserted into the plants, subsequently started to express the proteins in the leaves, tissues and in entire plant body, making the plant toxic to control the insects. However, these proteins are produced potential risks to the environment and human health; this has been recently focused due to the release of transgenes (Nap et al., 2003). In cultivated soil, the insecticidal crystal proteins can enter from the genetically modified (GM) crop through root exudates. Transformed genes from GM crops may interact with other organisms that induce the changes to environments, due to the existing and increasing risks, which may change the non-toxic organism to toxic organism, further, it is very difficult to eliminate because of the altered genome based structural functions (Conner et al., 2003). And it also altered the soil chemical profiles, which may be considered as early and sensitive indicators of soil quality as well as to measure the soil microbial activity. More assessments are needed to determine the effects of *Bt* genes on soil ecosystems and extended to soil organisms. Even though, GM crops are as animal feed in several countries like Europe and Japan (Chowdhury et al., 2003). Prior to the release of Bollgard I and II cottons in market, the food, feed and environmental safety were evaluated by standard regulatory agencies (Purcell and Perlak, 2004). However, *Bt* cotton has some risks on environment and to living organisms. A very few *in vitro* and *in vivo* research on the cry proteins were reported so far based on safety assessment and impact of *Bt* crops over living organisms. In

the present study to revealed the activity of a range of enzymes, which is related to the nutrient cycle and determined the persistence of insecticidal crystal proteins expression in Bollgard II and SVPR2 cotton rhizosphere soils.

MATERIALS AND METHODS

Soil Preparation and Cotton Lines: Before the nursery experiment, ~9kg of soils which contains red, sandy and clay soils and cow dung in 1:1:1:1 ratio with bioinoculants of Azospirillum and phosphate solubilizing bacteria (PSB) were placed in each black color polythene bags and then the soils were saturated with tap water and equilibrated for one week before sowing cotton seeds. This study includes various treatments for both inoculants and two different cotton lines were used as *Bt* cotton (Bollgard II) and non-*Bt* (SVPR2) from Monsanto. The experimental polythene bag soils were watered wherever necessary with tap water to maintain soil moisture for cotton growth and do not apply any insecticidal to the plants for the control of Boll worms.

Soil Sampling: The two different bioinoculant strains of *Azospirillum lipoferum* (N_2 fixer) and *Bacillus megaterium* (PSB) were obtained from Bannari Amman Institute, Tamil Nadu, India. After 120 days of cotton growth, the rhizosphere soil was collected from experimental polythene bags. The collected soils were grounded, passed through a 1mm sieve, and stored at -20ºC before analysis of enzymatic levels and determination of Cry1Ac and Cry2Ab proteins.

Dehydrogenase Enzyme: Dehydrogenase enzyme level was estimated (Casida et al., 1964). Six grams of experimental rhizosphere soil samples were incubated for 24h at 37ºC in 1ml of 3% 2,3,5-triphenyl tetrazolium chloride (TTC), 0.2 g CaCO3, 1ml of 1% sucrose solution and 2.5ml distilled water. The sample was then blended with 50ml of ethanol and centrifuged at 2000 rpm for 10 min. The red colour 2,3,5-triphenyl tetrazolium formazan (TPF) supernatant was measured spectrophotometrically at 485nm.

Phosphatase Enzyme: One ml of 1% sodium glycerolphosphate, two drops of toluene and 4ml of distilled water were added to 10g of rhizosphere soil and incubated at 37ºC for 24h. After incubation, 1ml of supernatant was added with 1ml Folin–Ciocalteu reagent, 1ml of distilled water and 2ml of 20% sodium carbonate were added and boiled for 1min and read calorimetrically at 485nm for quantification of total phosphatase levels (Askin and Kızılkaya, 2006).

Urease Enzyme: For 0.25 ml of toluene, 0.75 ml of citrate buffer (pH 6.7) and 1 ml of urea substrate solution were added to 1g rhizosphere soil and incubated at 37ºC for 24hr. The formation of ammonium was determined spectrophotometrically at 578 nm (Hoffmann and Teicher, 1961).

Invertase Enzyme: Experimental rhizosphere soil sample invertase level was determined (Frankenberger and Johanson, 1983). One gram of soil was incubated with buffered sucrose solution and toluene at 37°C for 24h. After incubation the mixture was filtered and the filtrate was used for analysis of reducing sugars.

Protease Enzyme: Protease enzyme level was determined by the modified Toni method (Yang and Huang, 1994). To determine the protease level it involves the colorimetric assay of casein digestion. The assay was carried out with reaction mixture, containing 1ml of 1% casein in 50mM Tris Hcl buffer (pH 10.5) and 1g of soil, was incubated at 37°C for 1h. The reaction was stopped with 3ml of 5% trichloroacetic acid (TCA) and the suspension was centrifuged at 8000rpm for 10min Optical density was measured at 470 nm.

Extraction of Cry Proteins in *Bt* Cotton Leaves (Bollgard II): Experimental *Bt* cotton leaves Cry1Ac and Cry2Ab protein were measured by ELISA. *Bacillus thuringiensis* cotton leaves were washed with sterile double distilled water to remove dust particles. And 5 g of leaves were grounded in liquid nitrogen using a mortar and pestle. Approximately 45 mg of crushed plant tissue samples were placed into a 1.5ml microfuge tube (Eppendorf) and mixed for 3sec with extraction of phosphate buffered saline tween-20 (60mM PBS–pH 7.2, 150mM Nacl, 3Mm KCl, 0.05% Tween 20) using plastic pestles. The resulting suspension was centrifuged and the clarified supernatants used for ELISA. 50 µl of the extracted protein samples were added to the test wells.

Extractions of Cry Proteins in Rhizosphere Soil: On 250 mg of experimental rhizosphere soil sample was taken in eppendorf tube. Protein extracted from soil in 500µl of extraction buffer (Borate buffer: 50mM Borate–pH 10.5) was provided with the ELISA kit and homogenized in a vortex at maximum speed. The soil suspensions were incubated for 60 min at room temperature and centrifuged at 16 000 × g for 10 min at 15°C. Supernatants were concentrated five fold by ultrafiltration with Microcon YM-10 centrifugal filter devices (Millipore) before ELISA. The level of protein was determined by adding 50 µl of the extracted soil proteins to the test wells. To serve as reference, standard Cry1Ac and Cry2Ab in the range of serial dilution 5 to 160ng/g were added to the non-*Bt* soil samples.

Immunological Assay: The anti-Cry 1Ac or anti-Cry 2Ab anti-body conjugated with horse radish peroxidase of 50 µl was added to each well of the plate. Buffer blank, standards, positive and negative control were also added to each well and incubated at 37˚C for 60 min. The wells were washed with 250 µl of 1X wash buffer (1X extraction buffer), and this procedure was repeated for 3 times. Excess wash buffer was removed from the plate. 100 µl of substrate buffer (100mg tetramethyl benzidine, 100mg of 50mM acetate buffer pH 4.0) was added to each well. The

plate was incubated in dark for 15 min at room temperature. 100µl of stop solution (0.1N H_2SO_4) was added to each well. The absorbance was measured at 450 nm.

Statistical Analysis: All obtained data were analyzed statistically with the standard procedure (Gomez and Gomez, 1984). And it was expressed in the arithmetic means, i.e., ± standard deviation and one way ANNOVA for triplicate of each treatment. Statistical analysis was performed with SPSS 17.0.

RESULTS AND DISCUSSION

Bacillus thuringiensis (*Bt*) and non-*Bt* cotton plants were maintained for 120 days with treated bioinoculants. The experimental rhizosphere soil which is surrounded the root with a narrow region and influenced by many physical, chemical and biological properties that may alter the growth of the plant. When, the rhizosphere soil was maintained properly, the whole ecosystem health can be preserved. So the releases of substances from root as exudates are potentially risk to the soil environment and recent findings also focused on soil impacts. Profiles of soil enzymes are indicate the direction and strength of all kinds of biochemical processes in soil and act as key biological indicator of soil. Soil enzymes play a vital role in energy transfer mechanism, organic matter decomposition, nutrient cycling and crop productivity (Kumar et al., 1992). In the present study, the enzymes involved in the nitrogen, phosphorous, carbon and sulphur cycling were analyzed in the rhizosphere soil compared between *Bt* and non-*Bt* cotton soils are selected for the activities after postharvest.

According to the soil enzymes, the dehydrogenase levels in *Bt* cotton on phosphate solublizing bacteria treated soils, non-*Bt* and *Bt* cotton has been showed high, in earlier report also matched with our results that dehydrogenase enzyme level between *Bt* and non-*Bt* cotton rhizosphere was significantly ($P<0.05$) high in *Bt* cotton rhizosphere soil (Usha Mina et al., 2011) because it is respiratory chain enzymes, which play the important role on the energy production of organisms, the abundant survival of microorganism for the reason of the high activity and also found that dehydrogenases and urease levels could not influence the soil grown under greenhouse (Jastrzebska and Kucharski, 2007). Phosphatase and urease levels were responsible for soil microbial respiration, according to the urease enzyme in Azospirllum treated soil had showed higher than PSB. Previous report also showed that urease level has been changed by the addition of organic waste to soil (Goyal et al., 1999) and in many reports suggested that there are changes in urease level due to microorganisms in soil (Hadas et al., 2004). However, the phosphatase enzyme for the *Bt* and non-*Bt* cotton were showed very similar in between Azospirillum and phosphate solublizing bacteria inoculants rhizosphere soils (Fig.1 A & B).

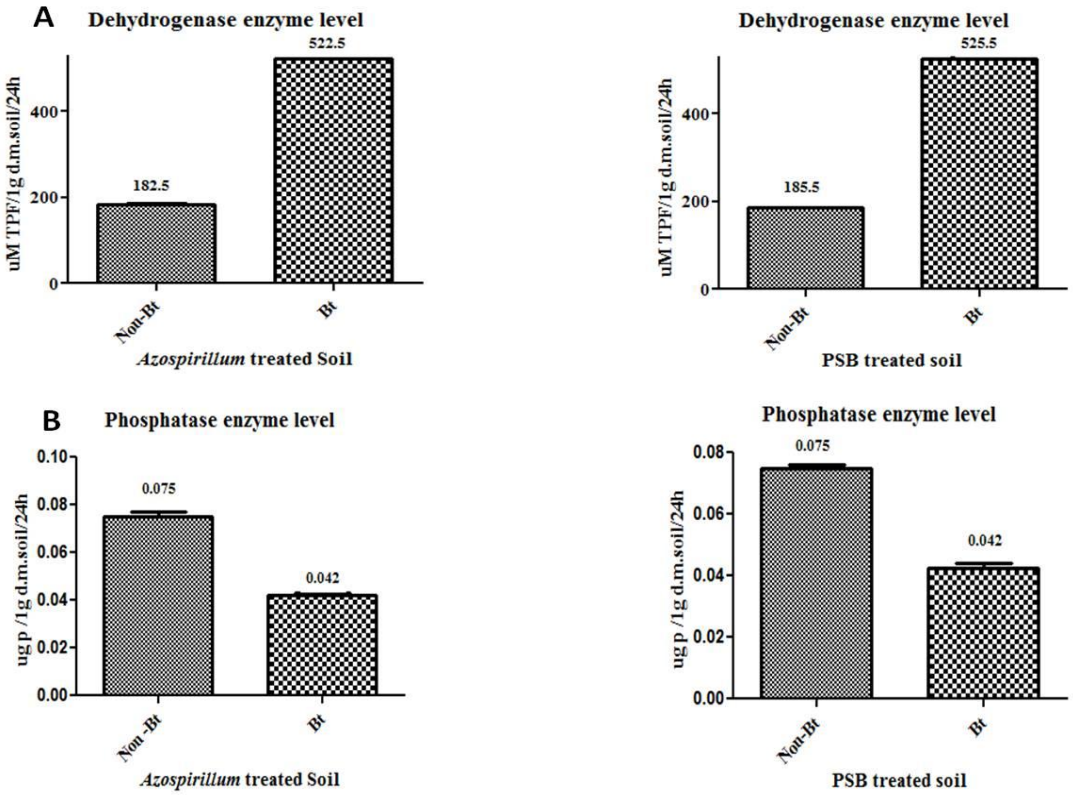

Figure. 1 Enzyme levels between bioinoculant treated rhizosphere soils for Non-*Bt* and *Bt*. **A:** Dehydrogenase enzyme; **B:** Phosphatase enzyme with significant at $p<0.05$ according to one-way ANNOVA

Our result was coinciding with (Gasco et al., 2004), that phosphatase having lower on its activity, while urease was stable and inversely proportional to each other. Previous studies have been stated that the effects of ammonium and nitrate are the major source for nitrogen on urease production in soils, which could alter the organic carbon. Invertase in *Bt* and non-*Bt* cotton soils were not showed any significant differences (Fig. 2 A & B).

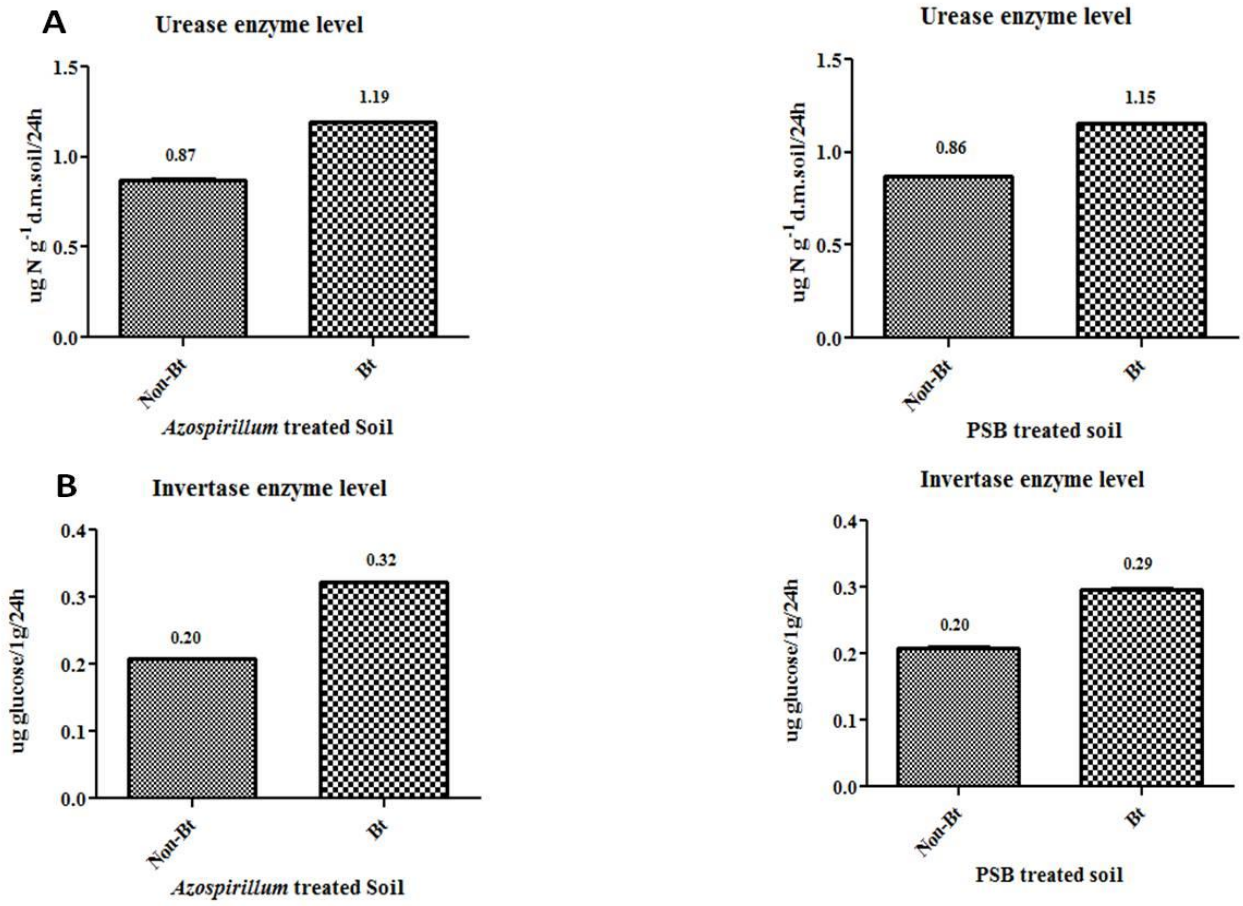

Figure. 2 Enzyme levels between bioinoculant treated rhizosphere soils for Non-*Bt* and *Bt*. **A:** Urease enzyme **B:** Invertase enzyme with significant at $p<0.05$ according to one-way ANNOVA

In the case of protease, *Bt* cotton soils were showed maximum activity in Azospirillum than PSB applied soil (Fig.3). In this present study also showed that there were not significant differences between Azospirillum and phosphate solublizing bacteria treated soil activities of dehydrogenases, phosphatase, urease, invertase and protease, the mean difference is significant at $p > 0.05$ level. Altogether, the only few enzyme levels were significant differences observed between *Bt* and non-*Bt* cotton, which is indicating that there are no harmful effects on soil enzyme levels in the rhizosphere. Similarly (Shen et al. 2006) have revealed that there was no consistent significant difference in between the soils of *Bt* and non-*Bt* cotton activities of some enzymes like urease, alkaline phosphatases, dehydrogenases, phenol oxidase and proteases. However, the related results were observed insignificant difference ($P<0.05$) in enzyme levels between *Bt* and non- *Bt* cotton rhizosphere during crop growth (13). ELISA has been used for qualitative and quantitative method to detect transgenic protein present in the plant tissues, leaves and in the environment (Elena et al., 2010).

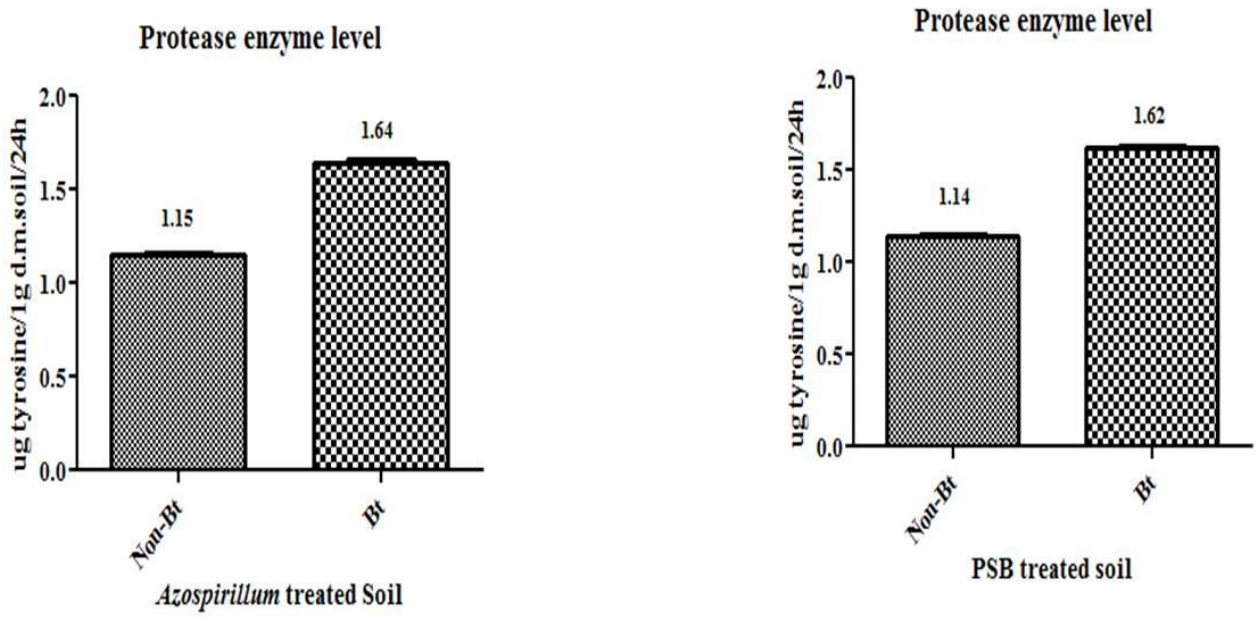

Figure. 3 Protease Enzyme levels between bioinoculant treated rhizosphere soils for Non-*Bt* and *Bt* with significant at $p<0.05$ according to one-way ANNOVA

Bacillus thuringiensis bollgard II cotton variety was expressed at level of 60µg/ml in postharvest rhizosphere soil. Though, Cry2Ab had expressed negligible amount, when compared to positive and negative control and non-*Bt* cotton rhizosphere soil with mean difference on significant at $p > 0.05$ level. Bollgard-II ranges between 14.0 and 83.0 µg /g fresh weight in leaves, and 5.5–62.0 µg/g fresh weight in fruiting parts (Greenplate et al., 2000), however the expression of Cry2Ab levels at >10 fold higher than Cry1Ac, which was coincide with the present study. Hence, *Helicoverpa armigera* in India is being uncovered to Cry1Ac independently and also Cry1Ac and Cry2Ab. It revealed that the usage of genetic engineering plants expressing single and dual *Bt* genes improved insect adaptation to pyramided plants (Zhao et al., 2005). Cry1Ac and Cry2Ab from rhizosphere soil protein were detected through ELISA. No expression in Cry1Ac but Cry2Ab had expressed very negligible amount, when compared with the positive and negative control and non-*Bt* cotton rhizosphere soil, although the level of protein in soil was not as much of 2.5ng/protein. It has been very less than the detection limit of procedure followed. This study also had showed that absence of protein expression from postharvest rhizosphere soil, because it was previously profound that half life for Cry1Ac protein in soil ranged from 18 to 40 days and it dependent upon soils too (Palm et al., 1994). Though, significant differences was not observed in Cry1Ac among the treatments (Chen et al., 2011) and Cry1Ac expression has been declines extremely towards end of the season. This may be due to some proteins which were protected from biodegradation that might have been expressed in the soil. Cry2Ab levels are at least 10 fold higher than Cry1Ac at any given point of time in any parts of the plant. The higher expression levels compensate for the lower insecticidal activity of Cry2Ab against bollworms (Kranthi et al., 2005). Though, previous report has clearly showed that *Bt* cotton of Cry1Ac was not high, when compared to non-*Bt*, the Cry2Ab had expressed nevertheless Cry1Ac proteins are not detected in their non-transgenic cotton line Azospirillum and phosphate solublizing bacteria in rhizosphere soil. The repeated cultivation of transgenic cotton had significantly harmful effects over microbial properties and enzyme activities as well as profiles in the rhizosphere (Chen et al., 2011). However, we cannot conclude that *Bt* crops as ecosafety for through this study.

CONCLUSIONS

Bacillus thuringiensis (*Bt*) cotton is a significant tool for farmers around the world. Based on nursery experiment with two different bioinoculants treated soil on *Bt* cotton, it was found that the absence of significance effects on rhizosphere soil profiles. So, the *Bt* cotton does not have any adverse effect on rhizosphere environments including soil biota.

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