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EFFECT OF Bt-TRANSGENIC COTTON ON SOIL BIOLOGICAL HEALTH

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ABSTRACT

Bt cotton are plants that have been genetically modified to express the insecticidal proteins *Cry 1 Ac* from subspecies of the bacterium, *Bacillus thuringiensis* israelensis (Bt), to control bollworm pest that feed on cotton. There is a persistent environmental concern that transgenic Bt-crops carry genes that have indirect undesirable effect to natural and agro–ecosystem function. We investigated the effect of Bt-cotton (with *Cry 1 Ac* gene) on several microbial and biochemical indicators in fields under sub-humid tropical condition. Twenty five fields were selected in the Vidarbha region, India, where Bt-cotton has been growing at least three consecutive years and side by side field of non-transgenic cotton is growing under clay to clay loam soil. Soil from a control (no-crop) treatment was also included from each area to compare the extent of adverse effect of Bt, if any. Samples were analyzed for actinobacteria, fungi and nitrifiers population, biomass carbon (MBC), biomass nitrogen (MBN), biomass phosphorus (MBP) and soil enzyme activities. The result revealed a significant decline in actinobacteria (17%), bacterial (14%) count as well as acid phosphatases (27%), phytase (18%), nitrogenase (23%) and dehydrogenase (12%) activities in Bt cotton compared with non-Bt cotton fields. Fungal and nitrifiers counts and esterase and alkaline phosphatase activities were not affected by the introduction of Bt-cotton in fields. However, significant decline between 8 and 9% in MBC and MBN was also noticed.

Keywords: Bt toxin, enzyme activities, microbial biomass, soil health, transgenic Bt-cotton, tropical sub-humid climate

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive, aerobic, spore-forming, rod-shaped bacterium that produces a parasporal, proteinaceous, crystalline inclusion during sporulation. This inclusion which may contain more than one type of insecticidal crystal protein (ICP), is solubilized and hydrolyzed in the mid gut of larvae of susceptible insects when ingested, releasing polypeptide toxins that eventually cause death of the larvae (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998). The ICPs have been classified on the basis of their structure, encoding genes, and host range and on the flagellar H-antigens of the bacteria that produce them (Höfte and Whiteley, 1989; *Crickmore et al.*, 1998). Numerous distinct crystal protein (*Cry*) genes have been identified that code for insecticidal proteins (Cry proteins). In general, the insecticidal properties of Bt are attributable to a crystalline protein (i.e. pro-toxin, or Bt-toxin) produced in the plant cells upon expression of the Cry genes. As long as the protein is free it is not toxic but when ingested by larvae of susceptible insect species (e.g., bollworm in cotton), it dissolves rapidly in the gut of the insect and is converted to a polypeptide toxin that induces toxemia to death (Höfte and Whiteley, 1989). Lepidopteran pests, particularly the bollworm complex, are a major constraint to increasing agricultural productivity. Thus, development of Bt-transgenic plants has

tremendous potential to facilitate the production of valuable agricultural crops throughout the world. There is a persistent environmental concern that transgenic Bt-crops carry genes that have indirect undesirable effects to natural and agro-ecosystem functioning.

Bt-cotton would be able to substantially reduce the amount of pesticides used and have better control over bollworm pests. This in turn would reduce cost of production and increase yields due to reduced damage from bollworms. Although there are advantages of the transgenic Bt-crops, there are also issues of public health concerns and soil microbial functioning because these usually carry genes and produce compounds foreign to the environment. Bt- toxin is produced in every part of Bt-plants, and Bt-toxin production is often higher in the later stages of crop growth. During crop growth, soil microorganisms come in direct contact with transgenic *Cry* endotoxin as it is released from Bt-crops in root exudates and from decomposing tissues (Motavalli *et al.*, 2004; Rui *et al.*, 2005). While Bt occurs naturally in soil, growth of transgenic Bt-crop causes a large increase in the amount of *Cry* endotoxin (approximately 0.25g ha^{-1}) in the agricultural systems (Blackwood and Buyer, 2004). Thus transgenic plants have the potential to modify the rhizosphere chemistry, or by altering plant residue quality (Dunsfield and Germida, 2004; Motavalli *et al.*, 2004; O'callaghan *et al.*, 2005). Any change to the quality of rhizosphere exudates can modify the biota composition in soil as well as their activity (Stotzky, 2004; Patra *et al.*, 2006) and may produce changes in microflora and microfauna (Wei *et al.*, 2006; Griffiths *et al.*, 2006).

India is a leading cotton producing country, and ranks fifth in world in terms of growing Bt-cotton (3.8 million hectares in 2006) (James, 2006) and 7.52 million hectares in 2009 (Karihaloo and Kumar, 2009) but until now there has hardly been any information from India about the impact of Bt-cotton on soil microbiological processes under field conditions. Unlike temperate regions most of the Indian soils are poor in soil fertility (organic C < 1.5%, N < 0.2%); thus soil microbiological and biochemical response to Bt-crops may differ from what have been reported elsewhere. In a long-term perspective, any further deterioration in soil ecosystem function due to increasing adoption of Bt-cotton may be disastrous to the Indian agriculture. Therefore, we examined the effect of Bt-cotton on soil microbial functioning under cotton growing fields in tropical agro-ecosystem. The major objective of this study was to compare the microbial load and microbiological activity of Bt and non-Bt plant in soil. The study shall enable the assessment of risks of crops expressing the *Cry 1 Ac* protein for no target soil organisms compared to no crop soil.

MATERIALS AND METHODS

Study area

The studies were conducted in Vidarbha region of India where cotton is intensively grown. Vidarbha is the eastern region of Maharashtra state made of Nagpur and Amrawati Division of India. It is located between 19°30' N to 20°45' N latitude and 78°46' E to 79°01' E longitude. Large basaltic rock formations exist throughout Vidarbha caused by the Deccan Lava Trap. The main cash crop of the region is cotton. The average annual rainfall is 1200 mm out of which 90% is received during June to September. The selected sites were Akola, Amrawati, Bhandara, Buldhana, Chandrapur, Garchiroli, Gondiya, Nagpur, Wardha, Washim, and Yavatmal.

Soil samples

Soil samples were collected from Bt cotton growing areas of Vidarbha from twenty five different sites where Bt cotton had been planted at least for the previous 3-5 consecutive years, which was compared with the adjoining fields where non-Bt cotton was growing during that period. Soil samples were also collected from the no-crop area of each site to compare and to know the physico-chemical and microbiological properties of the soil of that area. The history of Bollgard cotton growing at each sampling site is summarized as Table 1. The sampling was done in 2nd week of December, 2007 during

the crop harvest in the crop rhizosphere zone. In each year, all the cotton growing fields were cultivated one to two times during the growing season, and the plant stalks were first shredded and then tilled into the soil at the end of each season immediately following harvest of cotton lint and seeds. At the time of sampling, a majority of plant residues incorporated into the soil in the previous seasons had mostly decayed.

At each of the sampling sites, five core samples, each 15 cm deep and 7.6 cm in diameter, were randomly taken from Bt cotton, non- Bt cotton and no-crop field. The distance between each core sample within the same field ranged from 15 to 40 m. All samples were stored at 4^{o}C immediately after sampling until analysis. Soil samples were analyzed within three weeks. The gravimetric moisture content was determined immediately.

Soil analysis

Microbial biomass carbon (MBC) was estimated by the chloroform fumigation extraction method (Jenkinson and Ladd, 1981; Bremner and Kessel, 1990), and microbial biomass nitrogen (MBN) by the modified fumigation extraction procedure of Brookes *et al.* (1985). Chloroform fumigation followed by $NaHCO_3$ extraction was employed for determining microbial biomass P (MBP) (Brookes *et al.*, 1982).

Dehydrogenase activity was assayed as described by Tabatabai (1982). The soil samples were incubated with 2,3,5 triphenyl tetrazolium chloride, and triphenyl formazon produced was determined at 485 nm. Esterase activity was determined as described by Schnürer and Rosswall (1982) using the substrate fluorescein diacetate, 3,6'-diacetyl fluorescein (FDA) into green colour fluorescein, and quantifying the product of reaction at 490 nm. Acid and alkaline phosphatases were assayed as reported by Tabatabai and Bremner (1969) using a acetate buffer (pH 5.4) and a Na-tetraborate-NaoH buffer (pH 9.4), respectively. Phytase activity was assayed by measuring inorganic phosphate (P_i) hydrolyzed from sodium phytate in acetate buffer (pH 4.5) incubating at 37^{o}C for 1 h (Ames, 1966). Nitrogenase activity was estimated as described by Larue and Kurtz (1972). Colony forming unit (CFU) of fungi, bacteria and actinobacteria was enumerated by standard serial dilution and pour plate method as described by Alexander (1982). The population of nitrifying bacteria was estimated by MPN method (Alexander and Clark, 1985).

The pH and electrolytic conductivity (EC) of soil samples were determined (soil: water ratio of 1:2.5) by a glass electrode and conductivity bridge, respectively (Jackson, 1967). Mechanical analysis, organic C, N, available P and K and moisture contents were estimated as described by Jackson (1967).

Microsoft Excel 2000 was used in statistical processing of the data. The least significant difference was calculated as described by Sokal and Rohlf (1981).

RESULTS

Soil characteristics

The used soils were alkaline in reaction, non-saline, with moderate water holding capacity, low moisture status, medium to high available phosphorus and low to medium K (Table 1 and 2). Microbiological activity was in medium range. The soil colour was black mainly derived from basalt rock, medium to heavy in texture with textural class clay to clay loam. There was no difference in soil characteristics before growing Bt or non-Bt cotton crops.

Microbial population

Microbial population was quantified from soil samples collected within Bt-cotton fields, non-Bt cotton fields or no-crops fields of twenty five different sites. From average values, it has been observed that bacteria (14%) and actinobacteria (17%) counts were significantly reduced under Bt cotton cultivation, although still much higher than that observed under no crop land (Table 3). Both counts of nitrifiers (4%) and fungi were not significantly affected compared to non-Bt cotton field.

Table 1: History of Bt-cotton planting at different sampling site in Vidharba region of India

Sampling District	No. of sampling fields	Soil type	Years of Bt-cotton grown prior to sampling
Akola	2	Clay to clay loam	2004-2006
Amrawati	3	Clay Loam	2003-2006
Bhandara	1	Clay	2004-2006
Buldhana	2	Clay loam	2004-2006
Chandrapur	3	Clay to clay loam	2003-2006
Garchiroli	3	Clay loam	2003-2006
Gondiya	2	Clay loam	2004-2006
Nagpur	3	Clay to clay loam	2002-2006
Wardha	1	Clay loam	2003-2006
Washim	1	Clay loam	2004-2006
Yavatmal	4	Clay to Clay loam	2003-2006

Table 2: Average enzyme activities, microbial biomass and physiochemical properties of Vidarbha soils under study area

Parameters	Non-Bt cotton	Bt cotton
pH	7.7±0.19	7.8±0.10
EC (dSm^{-1})	1.0±0.09	1.0±0.06
Sand (%)	31-38	31-39
Silt (%)	22-28	23-28
Clay (%)	33-37	32-38
Water holding capacity (%)	66.7±0.72	66.6±0.93
Moisture (%)	4.8±0.21	4.8±0.13
Organic carbon (%)	0.8-1.5	0.9-1.4
N (%)	0.2±0.04	0.2±0.04
Available P (mg kg^{-1})	45.2±18.58	46.1±19.65
Available K (mg kg^{-1})	60.7±11.92	63.0±13.84
Dehydrogenase (p kat g^{-1})	2.7±0.08	2.7±0.07
Acid phosphatase (EU × 10^{-4})	10.0±0.15	9.8±0.21
Alkaline phosphatase (EU × 10^{-4})	14.0±0.14	14.1±0.17
Phytase (EU × 10^{-4})	409.4±41.21	405.3±47.20
Esterase (EU × 10^{-4})	103.3±5.90	103.5±5.94
Microbial biomass (μg g^{-1})	502.6±15.25	498.7±18.32

EU = Enzyme unit

Microbial biomass C, N and P

From the average value of twenty five soil samples it has been observed that soil under Bt-cotton cultivar showed significantly less (between 8 and 9%) microbial biomass C, N and P than non-Bt cotton growing soils but they were higher than no-crop soils (Fig. 1). In general, microbial biomass C was reduced by 8.9%, microbial biomass N by 8.2% and microbial biomass P by 8.8%.

Table 3: Average value of microbial counts (N = 25) in Bt and non-Bt cotton soils

Microorganisms (CFU g^{-1})	Non-Bt cotton soil	Bt cotton soil	Increase (+) or decrease (-) from non-Bt cotton (%)	Level of significance	No-crop soil
Actinobacteria (× 10^5)	52.5±9.7	43.6±7.3	- 17.0	**	31.8±5.9
Bacteria (× 10^6)	85.9±8.9	73.7±8.5	- 14.2	*	59.1±6.2
Fungi (× 10^4)	31.1±6.9	31.3±5.2	+ 0.3	NS	19.8±3.4
Nitrifiers (×10^2)	19.7±2.5	18.9±2.4	- 4.1	NS	12.9±1.9

CFU – Colony forming unit; NS-non significant, * $p<0.05$; ** $p<0.01$

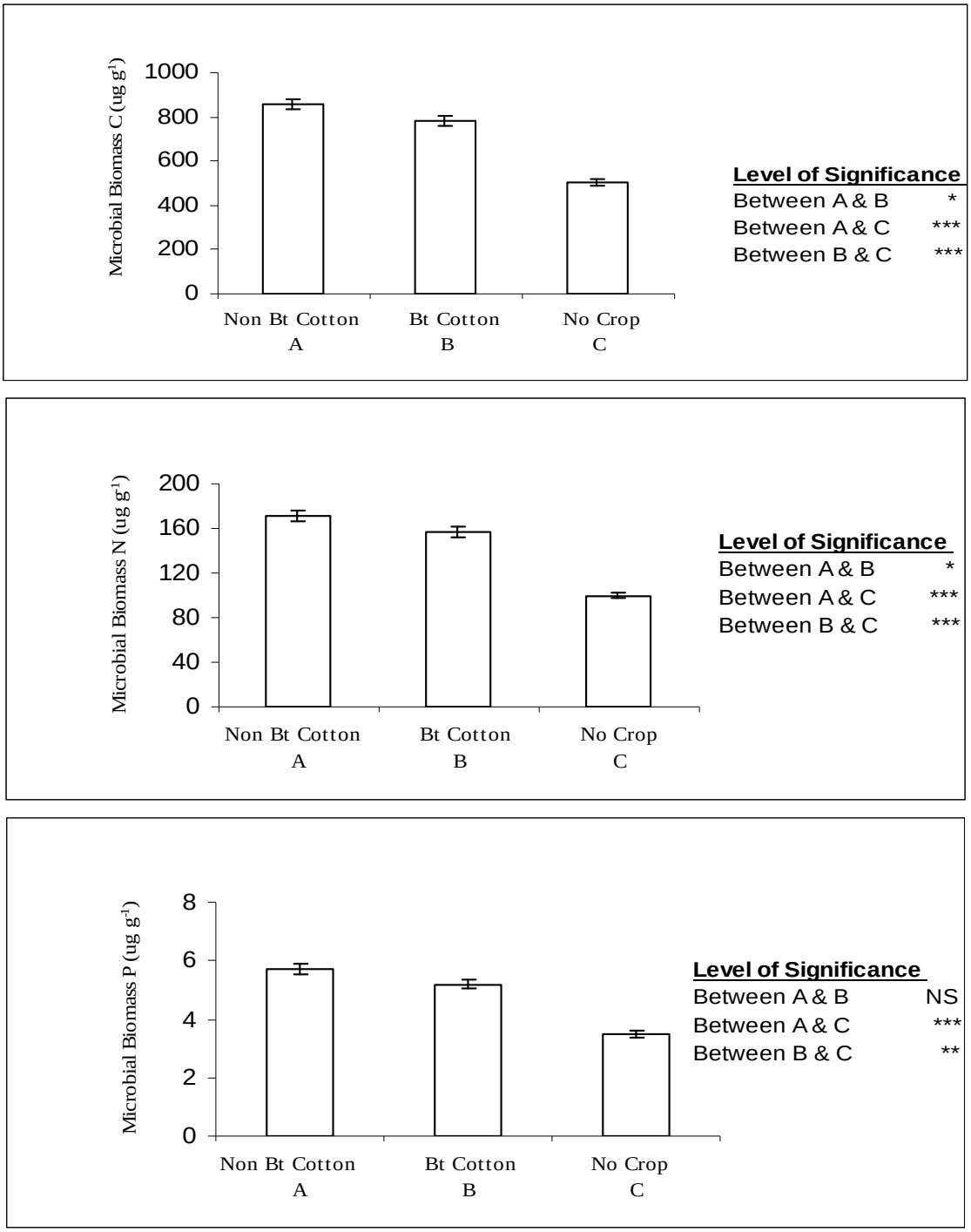

Fig. 1: Microbial biomass C, N and P of Bt-cotton, non-Bt cotton and no-crop soils [± indicates the standard errors of mean (* $p<0.05$; ** $p<0.01$; $p<0.001$; NS-non-significant)]

Enzyme activities

Dehydrogenase (10.3%, $p<0.05$), acid phosphatase (26.6%, $p<0.001$), phytase (18.1%, $p<0.01$) and nitrogenase activity (22.6%, $p<0.01$) declined significantly under Bt cotton as compared to values of soils under non-Bt cotton (Table 4). However, the enzyme activities were higher than those of soils

from no-crop fields ($p < 0.05$ and $p < 0.01$). No significant differences in esterase and alkaline phosphatase activities were noticed when compared between Bt-cotton with non-Bt cotton soils. In comparison of no-crop soils non-Bt cotton soils showed significantly higher average enzyme activities (dehydrogenase 26.1%; esterase 23.0%; acid phosphatase 32.3%; alkaline phosphatase 30.6%, phytase 28.9% and nitrogenase 30.8%).

Table 4: Enzyme activities in different cotton crop soils (average of 25 fields in each treatment)

Enzymes activity	Non-Bt cotton soil	Bt cotton soil	Increase (+) or decrease (-) from non-Bt cotton (%)	Level of significance	No-crop soil
Dehydrogenase (p kat g^{-1})	6.5±0.9	5.9±0.7	-10.3	*	4.8±0.7
Esterase (EU × 10^{-5})	45.2±3.4	41.8±2.8	-7.6	Ns	34.8±1.4
Acid Phosphatase (EU × 10^{-5})	29.8±1.9	21.9±1.0	-26.6	***	20.2±1.3
Alkaline Phosphatase (EU × 10^{-3})	32.2±3.2	31.9±3.0	-0.7	Ns	22.3±2.5
Phytase (EU × 10^{-5})	40.9±2.0	34.7±1.5	-18.1	**	29.1±2.1
Nitrogenase (nmol C_2H_4 h^{-1})	439±12.9	340±10.8	-22.6	**	304±9.7

ns – Non significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$: EU- Enzyme unit

DISCUSSION

In general cotton cultivation of at least three consecutive years has positively influenced 39% actinobacteria population, 31% bacterial population, 37% fungal population and 35% nitrifier population when compared with no crop soil to non-Bt cotton growing soils (Table 3). The less microbial C, N and P under Bt-cotton may be due to significant reduction in bacteria and actinobacteria population in soils. Microbial population and biomass is a small but very dynamic and essential component of nutrient cycling in soil. The change in root exudates and readily metabolizable C are perhaps the most influential factors contributing to less microbial biomass C in soils under Bt-cotton as root characteristics are significantly correlated with soil microbial biomass (Lynch and Panting, 1980).

Dehydrogenase and esterase activity reflects the total range of oxidative activities and thus are used as indicators of microbial activity in soil (Kumar and Tarafdar, 2003; Nannipieri *et al.*, 2003; Aseri and Tarafdar, 2006). Wu *et al.* (2004) have reported reduced dehydrogenase activity during the decomposition of straws from Bt-transgenic rice cultivars under flooded conditions in China. However, Shen *et al.* (2006) observed no differences in dehydrogenase activity between in the rhizosphere of Bt-cotton and that of non-Bt cotton. Differences in soil dehydrogenase or esterase activity are also indicative of variable amounts of labile organic C inputs (Nannipieri *et al.*, 2003). Lower activity in Bt-system indicates that some of the soil microorganisms were killed or inhibited by the toxin and did not participate in the metabolic activities in the soil (Masto *et al.*, 2006), which was also supported in results as significant reductions in cultivable actinobacteria and bacterial populations under Bt-cotton growing soils.

Stotzky (2004) reported that CO_2 evolved from soil amended with ground Bt (*Cry 1 Ab*) corn biomass was significantly lower than that from soil amended with unmodified isogenic corn biomass. While the mechanism by which the presence of the *Cry 1 Ab* toxin might depress microbial activities are unclear (Rui *et al.*, 2005; Griffiths *et al.*, 2006), it is likely that the insertion of the *Cry 1 Ac* gene into cotton may alter either the amount and or composition of root exudates and these effects may reduce respiratory activity. It is also likely that the insertion of the Cry gene into the cotton genome alters the composition and conformation of same recalcitrant components in cotton such as lignin, cellulose and hemicellulose, which protects the associated polysaccharides from microbial decomposition (Saxena and Stotzky, 2001).

Phosphorus is an important nutrient for cotton crop. Acid phosphatase, alkaline phosphatase and phytase are the enzymes which mineralize organic P to inorganic P to be taken by plants in available

form (Tarafdar, 2001; Tarafdar *et al*., 2002). Plants can only secrete acid phosphatase and phytase, while alkaline phosphatase is generally microbial in origin (Tarafdar, 1989; Yadav and Tarafdar, 2004). Our result indicates that Bt cotton affects the acid phosphatase and phytase secretion by the cotton roots. Nitrogenase activity is involved in N fixation and lower the activity indicates negative effect of Bt-rhizosphere condition on the bacterial communities involved in nitrogen fixation. As further support for this, we also found a reduced bacterial population (Table 3).

Conclusions: This study demonstrated that growth of Bt-cotton has a significant impact on some counts of microbial group (actinobacteria, bacteria), and biochemical indicators of the soil microbial communities such as, MBC, MBN, MBP as well as on a range of soil enzyme activities (acid phosphatase, phytase, dehydrogenase). However, the effect was not so alarming as the growing fields showed still significantly higher values than no-crop soils after three years of continuous Bt-cotton cropping. Future research should evaluate of continuous growing of Bt-cotton for several years in the same field might affect adversely the soil microbial function. It is also important to measure laccase activities of soils to see if there are changes in the degradation of lignin whose content is sometimes affected by genetic manipulation of plants.

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