Impact of transgenic cottons expressing c*ry1Ac* on soil biological attributes

K. Velmourougane, A. Sahu

Central Institute for Cotton Research, Indian Council of Agricultural Research, Nagpur, Maharashtra, India

ABSTRACT

Three transgenic Bt cotton hybrids (RCH-2 Bt, Bunny Bt and NHH 44 Bt) expressing *cry1Ac* gene were evaluated for their effects on soil biological, microbiological and diversity attributes at 0–15 cm and 15–30 cm soil depth under field conditions. At both soil depths, soil respiration rate and fluorescein diacetate hydrolysis were the highest in the soil under Bt cotton grown followed by non-Bt soil, and by the control bulk soil, indicating no adverse effects of Bt cotton on soil microbial activity. Urease and dehydrogenase activities, reflecting potentially available N and the oxidative metabolism in soil, respectively, also increased in the sequence no-crop variant < non-Bt soil < soil under Bt cotton at both soil depths. A similar trend was found with the soil microbial biomass carbon, microbial population and microbial diversity indices. These results suggest that cultivation of Bt cotton expressing *cry1Ac* gene may not pose ecological or environmental risk.

Keywords: Bt cotton; dehydrogenase; microbial biomass; urease; microbial diversity indices

There is a growing concern about cultivating transgenic cotton and its effects on general soil health. Most of the studies on impact of transgenic crops on soil properties carried out were restricted to contained or controlled conditions (Liu et al. 2005). Although some research has examined the environmental impacts of the 'aboveground' portion of transgenic crops, relatively fewer research effort has focused on the effects of these crops on soil microbes (Bruinsma et al. 2003) although no risk of growing transgenic Bt cotton on soil health is reported (Sun et al. 2007, Sarkar et al. 2009).

Genetically modified cotton genotypes incorporating a crystal (*Cry*) toxin producing *cry1Ac* gene derived from *Bacillus thuringiensis* (Bt), were introduced in India for commercial cultivation in the year 2002 (Morse et al. 2005). The transgenic crop, now popularly called Bt cotton, represents about 90% of cotton cultivated area in India. In India no comprehensive field study is available on the effects of growing transgenic cotton on soil biology. We evaluated the effects of growing three transgenic Bt cottons and their counterpart (non-transgenic cotton) on selected soil biological attributes under field conditions in deep vertisol.

MATERIAL AND METHODS

Experimental site and sampling. Three popular cotton hybrids (RCH-2, Bunny and NHH 44) of *Gossypium hirsutum* transgenic (expressing *cry1Ac* gene-Bt) and non-transgenic (no *cry1Ac* gene non-Bt), were evaluated with a randomized block design in triplicates under field conditions at the Central Institute for Cotton Research, Nagpur, India which is classified as Sub humid moist region with deep vertisols (Typic Haplusterts) receiving annual rainfall of 1200 mm. A control treatment was also included along with the main treatments in the form of bulk soil to assess the soil quality changes with no cotton crop. As both cultivars of cotton were alike, except for the presence of the Bt-gene, it was assumed that any differences in

soil ecological functions were attributable to the Bt-gene introduction in the cotton genotypes. The crop was raised under rainfed conditions during the rainy season (June–December) with 90 × 45 cm spacing. Normal agronomic practices were followed for raising the crop (basal fertilizer N:P:K: 90:45:45 kg/ha). Rhizosphere soils samples were collected 10 days before the harvest of crop at two depths; 0–15 cm and 15–30 cm from the experimental plots, and were labeled and transported back to the laboratory in polyethylene bags and stored at 4°C before analysis.

Soil biological analysis. Soil respiration was measured as the CO_2 evolved from moist soil, adjusted to 55% water holding capacity, and pre-incubated for seven days at 22–25°C with 10 mL of 1 mol/L NaOH. The CO_2 production was then measured by back titrating un-reacted alkali in the NaOH traps with 1 mol/L HCl to determine CO_2-C (Anderson 1982). Urease activity was determined according to the method described by Tabatabai and Bremner (1972), that involves the determination of the ammonium released by urease activity when 5 g of soil is incubated with 9 mL of 0.05 mol/L Tris (hydroxymethyl) aminomethane (THAM) buffer (pH 9.0), 1 mL of 0.2 mol/L of urea solution and toluene at 37°C for 2 h. The ammonium released was determined by a procedure involving treatment of the incubated soil sample with 2.5 mol/L KC1 containing a urease inhibitor (Ag_2SO_4) and steam distillation of an aliquot of the resulting soil suspension with MgO for 4 min. Dehydrogenase activity (DHA) in soils was determined following the method of Casida et al. (1964) by the colorimetric measurement of reduction of 2, 3, 5-triphenyl tetrazolium chloride (TTC). Each soil sample (10 g) was treated with 0.1 g $CaCO_3$ and incubated for 24 h at 37°C. The triphenyl formazan formed was extracted from the reaction mixture with methanol and assayed at 485 nm. FDA was measured following the method of Schnürer and Rosswall (1982) using 3, 6-diacetyl fluorescein as substrate and measuring the fluorescence at 490 nm. Soil microbial biomass was determined using the $CHCl_3$ fumigation-extraction method (Vance et al. 1987). Samples of moist soil (10 g) were used, and K_2SO_4-extractable C was determined using dichromate digestion. Microbial biomass carbon was calculated using the equation:

Biomass C = 2.64 EC, where: EC – (organic C in K_2SO_4 from fumigated soil) – (organic C in K_2SO_4 from unfumigated soil).

Soil microbiological and diversity analysis. Samples (10 g, fresh weight) were serially diluted in 90 mL Ringers solution up to 10^{-3} dilution and an aliquot of 1 mL of the aliquot was pour plated into selective media (nutrient agar for bacteria), Martin's Rose Bengal Agar for fungi, Ken-Knight and Munaier's Agar for actinomycetes and Buffered yeast agar for yeast. The plates were incubated at optimum temperature (28 ± 1°C for bacteria and yeast; 30 ± 1°C for fungi and actinomycetes) in triplicates. The functional groups of microbes were enumerated by following standard microbiological methods (Wollum 1982). The microbial colonies appearing after the stipulated time period of incubation (3 days for bacteria and yeast; 5 days for fungi; 7 days for actinomycetes) were counted as colony forming units and expressed as cfu/g. The culturable microbial diversity indices of Bt and non-Bt cotton grown soils were determined following standard methods (Hill et al. 2003).

Statistical analysis. Significant ($P < 0.01$ and $P < 0.05$) differences between Bt and non-Bt cotton on soil biological attributes were analyzed in the SAS programme (version 9.1). Tukey's multiple comparison tests were done to determine the differences between Bt and non-Bt cotton hybrids.

RESULTS AND DISCUSSION

Impact of Bt cotton on soil respiration and FDA hydrolysis. Soil respiration rate was significantly ($P < 0.01$) highest in the Bt cotton grown soil followed by non-Bt grown soil, and least in the control bulk soil without cotton at both the soil depths (0–15 cm and 15–30 cm) (Figure 1a). Soil respiration rate also varied significantly among the cotton hybrids of Bt and non-Bt. Among the cotton hybrids, RCH-2 recorded the highest soil respiration rate in Bt as well as in non-Bt cotton. The increased soil respiration rate with Bt cotton in our study is the outcome of higher root volume in Bt cotton compare to non-Bt cotton that have stimulated the microbial growth and activity by enhanced resource availability. FDA hydrolysis differed significantly between the Bt cotton and non-Bt cotton at both the soil depths (0–15 cm and 15–30 cm). While the control bulk soil showed lesser FDA hydrolysis (Figure 1b), NHH 44 recorded higher FDA hydrolysis in Bt and non-Bt cotton. The higher FDA in Bt cotton soil indicates the

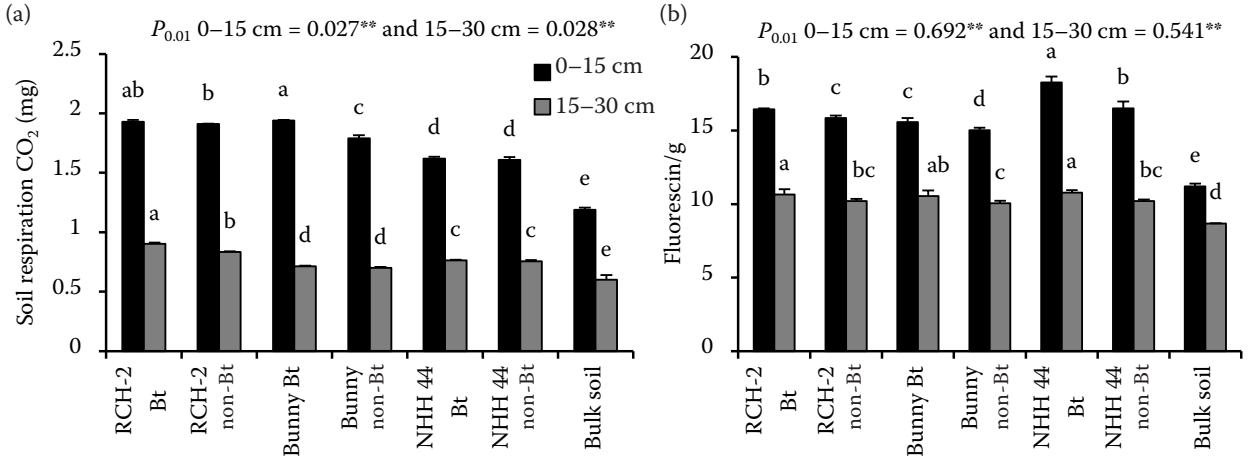

Figure 1. Effects of Bt and non-Bt cotton on biological attributes (a) soil respiration; (b) fluorescein diacetate hydrolysis. **$P < 0.01$. Error bars (± SD) with the same letters within the cotton genotypes do not differ significantly (Tukey's test, $P < 0.05$)

healthy microbial activity and no adverse effects of Bt cotton on soil microbial activity.

Impact of Bt cotton on soil urease and dehydrogenase activities. Soil enzymes were suggested as one of the potential biological indicators of soil quality because of their relationship to soil biology, ease of measurement, and rapid response to changes in soil management. Urease plays an important role in the efficient use of urea fertilizer in soils and the changes in urease activity is used as an indirect indicator of the variation in the pool of potentially available N in a soil. In our present study, the soils under Bt cotton had higher urease and dehydrogenase activities than under non-Bt or no-crop. Soil grown with Bt cotton recorded higher urease activity compare with non-Bt and no-crop treatments at both the soil depths (Figure 2a). Among the cotton hybrids, Bunny Bt recorded higher soil urease activity. The reason for increased urease activity in the Bt cotton rhizosphere compare to the non-Bt cotton and control bulk soil rhizosphere was the results of greater availability of organic C, nutrients and stimulated microbial activity attributable to better root spread and volume in Bt cotton. Urease activity was reported to be proportional to organic C distribution in each soil profile (Tabatabai 1977). Previously, Sun et al. (2007) observed higher urease activity by the addition of Bt cotton tissues in the soil. Usha et al. (2011) reported higher urease activity in Bt cotton cultivated soil and higher nitrate reductase, acid and alkaline phosphatase activities were also reported in the soil under Bt cotton cultivation (Sarkar et al. 2009). Bt cotton grown soil showed

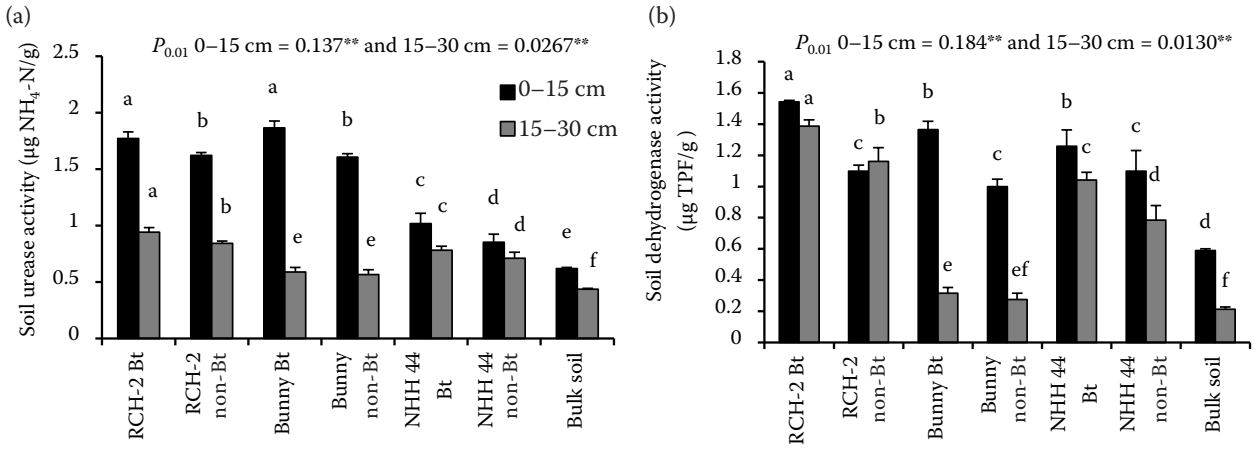

Figure 2. Effects of Bt and non-Bt cotton on soil enzyme activities (a) soil urease; (b) soil dehydrogenase. **$P < 0.01$. Error bars (± SD) with the same letters within the cotton genotypes do not differ significantly (Tukey's test, $P < 0.05$)

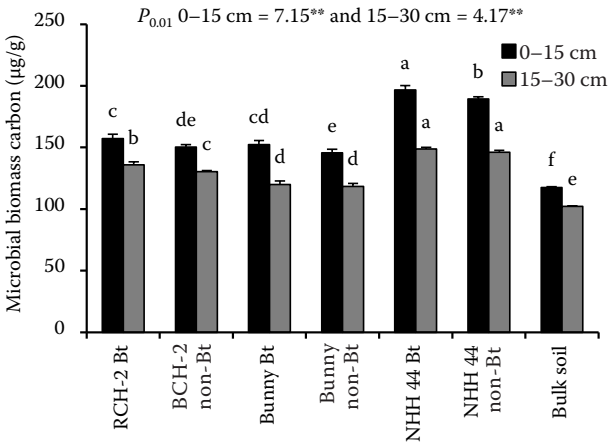

Figure 3. Effects of Bt and non-Bt cotton on soil microbial biomass carbon. **$P < 0.01$. Error bars (± SD) with the same letters within the cotton genotypes do not differ significantly (Tukey's test, $P < 0.05$)

significantly ($P < 0.01$) higher DHA as compared to non-Bt cotton grown soil at both the soil depths (Figure 2b). Among the cotton hybrids, RCH-2 followed by Bunny cotton recorded higher DHA in Bt cotton; while in non-Bt cotton, there was no much differences among the cotton hybrids at 0–15 cm depth. The higher DHA in Bt cotton grown soil is mainly attributed to the higher microbial activity stimulated by higher root density in Bt cotton compare with non-Bt cotton. DHA is considered as an indicator of the oxidative metabolism in soils and thus of the microbiological activity (Garcia et al. 1997) because it is linked to viable cells. Soil DHA reflects the total range of oxidative activity of soil microflora and, consequently it may be a good indicator of microbiological activity in the soil (Skujins 1976). Positive correlations between dehydrogenase activity and Bt cotton cultivation are also reported (Singh et al. 2013). DHA in soil depends on the content of soluble organic carbon (Zaman et al. 2002) and the increased organic matter in the surface soil horizon enhanced the soil enzyme activities. Studies by Furczak and Joniec (2007) showed that stimulation of DHA was accompanied by an increase in the number of the microbial groups and improvement in other living conditions (aeration and moisture).

Impact of Bt cotton on soil microbial biomass carbon. Soil under Bt cotton hybrids had on average significantly ($P < 0.01$) higher amounts of MBC (168.7 and 134.9 µg/g) compared with the non-Bt (161.87 and 131.6 µg/g) and bulk soil (117.57 and 102.2 µg/g) at 0–15 cm and 15–30 cm soil

Table 1. Effects of Bt and non-Bt cotton on soil culturable microbial population

Cotton hybrids	General microflora (cfu × 10^3/g)								Functional microflora (cfu × 10^3/g)							
	bacteria		fungi		yeast		actino-mycetes		*Azotobacter* spp.		*Beijerinckia* spp.		PSM		fluorescent pseudo-monads	
	soil depth (cm)															
	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30
RCH-2 Bt	40.3^{a}	32.0^{a}	4.3^{a}	2.3	15.0^{b}	10.0^{ab}	10.7^{a}	6.0	2.3	1.3	3.3	2.6	1.6	1.0	5.3^{a}	1.3
RCH-2 non-Bt	32.6^{b}	25.6^{b}	3.3^{ab}	1.7	12.0^{bc}	7.0^{bc}	7.0^{abc}	3.6	2.0	1.0	2.3	1.6	1.3	1.3	3.0^{b}	0.6
Bunny Bt	34.3^{b}	25.6^{b}	1.6^{b}	2.0	12.0^{bc}	7.7^{bc}	6.3^{bc}	2.6	1.6	1.6	4.6	1.0	1.3	1.0	2.3^{bc}	1.6
Bunny non-Bt	27.3^{c}	23.3^{b}	1.3^{b}	1.0	8.6^{cd}	4.3^{c}	4.3^{c}	1.3	1.3	1.3	3.3	1.0	1.3	1.0	1.7^{bc}	1.6
NHH 44 Bt	36.0^{b}	28.6^{ab}	4.3^{a}	2.7	20.8^{a}	12.0^{a}	10.7^{a}	4.6	3.0	0.6	4.3	1.0	0.6	0.6	1.0^{c}	0.6
NHH 44 non-Bt	23.0^{d}	24.6^{b}	3.6^{ab}	2.0	15.7^{b}	8.0^{abc}	9.7^{ab}	4.0	2.0	0.6	3.6	0.6	1.0	1.0	1.3^{c}	1.0
Bulk soil	18.5^{e}	14.0^{c}	1.3^{b}	1.1	6.7^{d}	4.3^{c}	3.0^{c}	2.1	1.0	0.5	2.0	0.3	0.3	0.3	1.0^{c}	0.6
SEM	1.27	2.61	0.97	0.81	1.83	1.44	1.41	1.39	0.83	0.67	1.26	0.51	0.50	0.54	0.97	0.70
$P < 0.05$	3.99	5.9	2.39	ns	4.14	4.17	4.02	ns	ns	ns	ns	ns	ns	ns	1.66	ns
$P < 0.01$	5.55	ns	ns	ns	5.75	ns	5.58	ns	ns	ns	ns	ns	ns	ns	2.31	ns

SEM – standard error of mean; ns – non significant. All values are the mean of three replicates. Means followed by the same letter within a column do not differ significantly (Tukey's test, $P < 0.05$). PSM – phosphorus solubilising microbes

Table 2. Effects of Bt and non-Bt cotton on culturable microbial diversity indices

Cotton hybrids	Shannon-Weiner index (H')		Simpson's index of dominance (D)		Shannon Evenness (E)		Simpson's Evenness (E)	
	0–15	15–30	0–15	15–30	0–15	15–30	0–15	15–30
RCH-2 Bt	2.67 ± 0.05^{ab}	2.48 ± 0.09	0.40 ± 0.01^{ab}	0.45 ± 0.03	0.89 ± 0.02^{abc}	0.83 ± 0.03	0.54 ± 0.01^{abc}	0.50 ± 0.02
RCH-2 non-Bt	2.58 ± 0.02^{abc}	2.32 ± 0.23	0.42 ± 0.01^{bc}	0.50 ± 0.07	0.86 ± 0.01^{bcd}	0.76 ± 0.09	0.52 ± 0.01^{bcd}	0.46 ± 0.06
Bunny Bt	2.45 ± 0.18^{bc}	2.29 ± 0.25	0.47 ± 0.05^{c}	0.51 ± 0.08	0.81± 0.06^{cd}	0.75 ± 0.10	0.49 ± 0.05^{d}	0.45 ± 0.07
Bunny non-Bt	2.36 ± 0.18^{c}	2.04 ± 0.18	0.48 ± 0.04^{cd}	0.59 ± 0.06	0.78 ± 0.07^{d}	0.65 ± 0.08	0.47 ± 0.04^{d}	0.37 ± 0.06
NHH 44 Bt	2.76 ± 0.05^{a}	2.50 ± 0.19	0.38 ± 0.01^{ab}	0.44 ± 0.05	0.92 ± 0.02^{ab}	0.83 ± 0.07	0.57 ± 0.01^{ab}	0.51 ± 0.04
NHH 44 non-Bt	2.75 ± 0.01^{a}	2.37 ± 0.15	0.35 ± 0.00^{a}	0.47 ± 0.04	0.95 ± 0.00^{a}	0.78 ± 0.06	0.59 ± 0.00^{a}	0.48 ± 0.04
Bulk soil	2.39 ± 0.02^{c}	2.20 ± 0.18	0.46 ± 0.01^{c}	0.53 ± 0.07	0.79 ± 0.01^{d}	0.72 ± 0.07	0.49 ± 0.01^{cd}	0.43 ± 0.06
SEM	0.04	0.05	0.01	0.01	0.01	0.02	0.01	0.01
$P < 0.05$	0.234	ns	0.06	ns	0.08	ns	0.05	ns
$P < 0.01$	ns	ns	ns	ns	ns	ns	ns	ns

SEM – standard error of mean; ns – non significant. All values are the mean of three replicates ± SD. Means followed by the same letter within a column do not differ significantly (Tukey's test, $P < 0.05$)

depths, respectively (Figure 3). Among the cotton hybrids, NHH 44 showed higher MBC in both Bt and non-Bt. The increased MBC in the soil grown with Bt cotton is attributed to higher root volume compared with non-Bt cotton. Possibly readily metabolisable carbon and nutrient availability at Bt cotton rhizosphere and differences in root exudates are perhaps the most influential factors contributing to increased microbial colonization and subsequent higher MBC in soils under Bt cotton. Earlier, Sarkar et al. (2009) reported a significant correlation between root volume of Bt cotton and soil MBC that supports the findings of Lynch and Panting (1980) that soil MBC increased with root growth and rooting density of the crop. Moreover, significantly higher population of different microbial groups was reported in field plots under transgenic alfalfa (*Medicago sativa* L.) (Donegan and Seidler 1999), cotton (Donegan et al. 1995), papaya (Wei et al. 2006), and maize (Griffiths et al. 2006).

Impact of Bt cotton on soil culturable microbial population and diversity indices. Bacterial and fungal population was significantly higher in Bt cotton grown soil compare with non-Bt soil at 0–15 cm depth, while at 15–30 cm fungal population showed no significance (Table 1). Yeast and actinomycetes population followed the similar trend as that of bacterial population. Among the cotton hybrids, RCH-2 recorded higher microbial population in Bt and non-Bt cotton. Except fluorescent pseudomonads, there were no significant differences ($P < 0.05$) in the population of functional microflora between *B*t and non-Bt cotton hybrids at both the soil depths. Bt cotton grown soil indicated higher microbial diversity indices compare with non-Bt cotton at 0–15 cm soil depth (Table 2). Among the cotton hybrids, NHH 44 showed higher microbial diversity indices in both Bt and non-Bt cotton. The increase in microbial population and diversity indicates no adverse effects of growing Bt cotton on soil microbial activity. The differences in the microbial population and diversity indices of Bt and non-Bt cotton hybrids may be attributed to variations in root exudates quantity, composition and root characteristics bring about by the genetic makeup of the cotton rather than expression of *cry* gene. Previous studies (Yan et al. 2007) have shown that the qualitative and quantitative differences in root exudation of Bt cotton could strongly influence the structure of microbial communities in the rhizosphere. Higher microbial populations in transgenic cotton grown soil were also reported by several workers (Shen et al. 2006, Kapur et al. 2010). Hu et al. (2009) based on their multiple-year cultivation showed that transgenic Bt cotton was not found to affect the rhizosphere functional bacterial population. Saxena and Stotzky (2001) did not observe any significant differences in the numbers of culturable bacteria, actinomycetes, and fungi in the rhizosphere of transgenic Bt and non-transgenic maize. Similarly, Brusetti et al.

(2005) detected no differences in the rhizosphere bacterial communities between transgenic Bt 176 maize and its non-transgenic counterpart.

Other authors, however, reported minor to significant effects of Cry proteins and transgenic Bt crops on microbial community structure in soil. Petras and Casida (1985) observed slight increase in populations of bacteria, actinomycetes, fungi, and nematodes after the addition of *B. thuringiensis* subsp. *kurstaki* to the soil and they inferred that the crystal proteins were used as a substrate by soil microbes. A significant but transient increase in the populations of culturable bacteria and fungi was observed in soil incorporated with leaves of Bt cotton (*Gossypium hirsutum* L.) expressing the *cry1Ac* protein (Donegan et al. 1995).

In conclusions, this study has demonstrated 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. Temporal variations observed between Bt and non-Bt cotton were attributable to differences in genetic makeup of the cotton hybrids rather than gene expression. Our results suggest that cultivation of Bt cotton expressing *cry1Ac* gene may not pose ecological or environmental risk.

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Corresponding author:

Kulandaivelu Velmourougane, Indian Council of Agricultural Research, Central Institute for Cotton Research, Nagpur 440010, Maharashtra, India
phone: + 91 07103 227 5549 Ext: 254, fax: + 91 07103 227 5529, e-mail: velicar@gmail.com