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Abstract

Growing areas under transgenic crops have created a concern over their possible adverse impact on the soil ecosystem. This study evaluated the effect of Bt-cotton based cropping systems on soil microbial and biochemical activities and their functional relationships with active soil carbon pools in Vertisols of central India (Nagpur, Maharastra, during 2012-2013). Culturable groups of soil microflora, enzymatic activities and active pools of soil carbon were measured under different Bt-cotton based cropping systems (e.g., cotton-soybean, cotton-redgram, cotton-wheat, cotton-vegetables and cotton-fallow). Significantly higher counts of soil heterotrophs (5.7-7.9 log cfu g^{-1} soil), aerobic N-fixer (3.9-5.4 log cfu g^{-1} soil) and P-solubilizer (2.5 -3.0 log cfu g^{-1} soil) were recorded in Bt-cotton soils. Similarly, soil enzymatic activities, viz. dehydrogenase (16.6- 22.67 µg TPF g^{-1} h^{-1}), alkaline phosphatase (240-253 µg PNP g^{-1} h^{-1}) and fluorescein di-acetate hydrolysis (14.6-18.0 µg fluorescein g^{-1} h^{-1}), were significantly higher under Bt-cotton-soybean system than other Bt- and non-Bt-cotton based systems in all crop growth stages. The growth stage-wise order of soil microbiological activities were: boll development > harvest > vegetative stage. Significant correlations were observed between microbiological activities and active carbon pools in the rhizosphere soil. The findings indicated no adverse effect of Bt-cotton on soil biological properties.

Keywords: Bt-cotton; Soil microbial activities; Soil carbon pools; Glomalin related soil protein, Vertisols

Introduction

One of the major apprehensions about genetically modified plants is that a continuous cultivation of these crops could impart undesirable consequences on the soil ecosystem (Turrini et al. 2015). Soil biota regulates a number of soil functions related to nutrient cycling, and any deviation in the quality of crop residue inputs through transgenic crop cultivation might potentially modify the microbial community dynamics and their functions (Zhang et al. 2016). Cultivation of nutrient exhaustive crops like Bt-cotton may lead to a rapid depletion of soil organic C and other essential nutrients, which might cause soil degradation (Lal 2015). Although intensive cultivation of Bt-cotton might add increased biomass to the soil, a negative C and N balance might occur due to rapid depletion of nutrients in such systems (Sarkar et al. 2008; Beura & Rakshit 2011). Further, intensive cultivation under mechanized farming cause rapid changes in native ecosystems that might cause easy oxidation of soil organic C (Awale et al. 2017).

The improvement in cotton productivity occurred because of a decrease in loss by bollworms due to the introduction of Bt-toxin gene in plants, and reduced cost incurred on plant protection chemicals (Ibrahim & Shawer 2014). In India, transgenic Bt-cotton are being cultivated in about 11.4 million hectares with an adoption rate of about 93%, and this represents approximately 36% of the global cotton area (James 2017). It has improved the cotton productivity, but information of its consequence on soil health sustenance is inadequate (Guan et al. 2016), especially under Indian conditions. Previous studies investigated this aspect under controlled conditions or in research experimental trials (Mina et al. 2008; Sarkar et al. 2008; Velmourougane & Sahu 2013), but realistic conditions in farmers' fields were ignored.

Some in vitro and in vivo studies showed that Bt-cotton plants contain Bt-toxin in leaves, stems and roots (Mina et al. 2008). The impact of Bt-toxin on soil microorganisms is either inconsistent

58 or negligible under various agro-climatic conditions (Kapur et al. 2010; Hu et al. 2013;
59 Velmourougane & Sahu 2013). However, the continuous growing of transgenic crops in the
60 same location might enhance the toxin's concentration to a level that might influence the
61 composition and activity of soil microbial communities and microbiological properties (Zhaolei
62 et al. 2017; Li et al. 2018). Existing literature present inconclusive data on this issue. For
63 example, changes in the soil microbial community structure associated with genetically modified
64 (GM) plants were temporary, and did not persist till the next season of canola cultivation
65 (Dunfield & Germida 2003). Under controlled pot experiment, Bt-cotton showed a positive
66 influence on most of the soil microbial indicators, such as microbial biomass C, N and P,
67 microbial quotient and a range of soil enzymatic activities in comparison to its non Bt isoline
68 (Sarkar et al. 2009). Similarly, a depth wise (0-15 and 15-30 cm) field study demonstrated that
69 the soils grown with transgenic Bt-cotton hybrids (RCH-2 Bt, Bunny Bt and NHH 44 Bt) showed
70 higher activities of microbial respiration and fluorescein di-acetate (FDA) hydrolysis than non
71 Bt-cotton counterparts (Velmourougane & Sahu 2013). Another field experiment showed that the
72 transgenic cotton (Bollgard-I, i.e., CIM-602, CIM-599, and non Bt varieties, i.e., CIM-591, CIM-
73 573) had no adverse effect on the viable counts of microbial population and enzymatic activity of
74 the rhizosphere soil (Yasin et al. 2016). Therefore, the effects of Bt-cotton on soil
75 microorganisms may be both variable and transient.
76 Soil- and plant-associated microbial communities are influenced not only by plant species and
77 transgene insertion, but also by geological/geospatial factors such as field site, soil type, clay
78 content and sampling time (Dunfield & Germida 2003; Icoz et al. 2008). Consequently, a lack of
79 scientific understanding still exists in relation to impact of genetically modified crops on below
80 ground ecological risk. Furthermore, very little is understood about the impacts of Bt-cotton

during its different physiological growth stages on soil microbiological attributes and cultivable microbial diversity under Indian field scenario. Such impacts under various historical background of cotton-based cropping systems is also not known. The present investigation therefore aims firstly, to assess the microbiological attributes and the cultivable diversity of beneficial microorganisms in the rhizosphere soil (Vertisols in central India) of Bt-cotton and non Bt-cotton during the crop's important physiological growth stages, and secondly, to establish the relationship between active soil carbon pools and biological attributes under Bt and non Bt-cotton crops.

Materials and Methods

Site characteristics

The experimental area is located between 20°42'52'' – 20°43'52''N and 78°55'33'' – 79 °6'54''E. The climatic condition falls under sub-humid, semi-arid, tropical zone in the Nagpur district of Maharashtra, India. The mean annual rainfall (1050 mm) at the location occurs mostly between June and October. April-May and December-January are the hottest (34°C) and coldest (20°C) months, respectively. The soil belongs to the hyperthermic family of Typic haplusterts. The cultivar (cotton hybrid RCH-2, Bunny Bt, Super maruti) containing Bt gene and its non-transgenic isoline were grown in randomized block design in triplicates under field conditions at the Central Institute for Cotton Research (CICR) experimental farm and also in ten farmers' fields in Central India, Maharastra. At the farmers' fields, Bt-cotton cultivars (cotton hybrid RCH-2, Bunny Bt, Super maruti 9632, Jai Bt, Ajit-11) were grown with their non Bt counterparts as a refugee crop in all cases. The farmers grew couple of additional varieties (Jai Bt and Ajit-11) in comparison to the CICR farm according to the availability of seeds supplied by the local dealers. The crop was raised under rain-fed condition during June 2012 to February 2013, with

90 x 45 cm plant-to-plant spacing. Fertilization was applied as per recommended agronomic practices (N:P:K 90:45:45 kg ha^{-1}). The rhizosphere soil samples were collected at three important growth stages of cotton (i.e., vegetative stage, boll development stage and harvest stage) from the CICR farms as well as farmers' fields. Soils grown with non Bt cotton isolines served as the control samples. In the CICR farm, a cotton-fallow cropping system was followed, while cotton-soybean, cotton-red gram, cotton-wheat, cotton-vegetable (as intercrop) and cotton-fallow cropping systems were followed in the farmers' fields. In both cases, these cropping systems were followed for a consecutive six years.

Soil sampling and analysis

The rhizosphere soil samples (0-20 cm depth) were collected in triplicate. Individual replication was composed of composite soil samples randomly collected from 10 different spots of each cropping system under Bt and non Bt-cotton. Samples were transported under refrigerated condition in sterilized polyethylene bags to the Soil Biology Laboratory of the Indian Institute of Soil Science, Bhopal, India. The spatial variability in the farmers' fields were eliminated by choosing the same sites where Bt and non Bt crops were grown. There was no variation in soil type, texture and climatic parameters (data not shown) under these field conditions.

Soil samples were processed, air-dried, ground and passed through a 2-mm sieve for chemical and microbiological analyses, and through a 1-mm sieve for carbohydrate carbon analysis.

The pH (1:2 soil: water suspension) and electrical conductivity (EC) of the soils (1:5 soil: water suspension) were measured by using a pH-EC meter (Model 1615, ESICO International, Parwanoo, India). Soil organic carbon was determined by the dichromate oxidation method. Available N content was estimated by conducting distillation of the soil with 0.32% $KMnO_4$ and 2.5% NaOH followed by measurement of evolved ammonia by alkali titration. Olsen's

127 extractant, 0.5M $NaHCO_3$ (pH 8.5), was used for measuring the soil available P by colorimetric
128 method using a spectrophotometer (CE 2031, Cecil Instruments Ltd., Cambridge, UK). Available
129 potassium (K) was extracted in neutral (pH 7.0) 1N ammonium acetate solution, and analysed by
130 a flame photometer (CL 378, Elico Ltd., Hyderabad, India).
131 Acid-hydrolysable carbohydrate (AHC) and water soluble carbon (WSC) in soils were
132 determined by standard methods (Supplementary Information; SI1 and SI2). The microbial
133 biomass C (MBC) in the pre-incubated soils (12 g dry weight equivalent) was determined by the
134 ethanol-free chloroform-fumigation extraction method (Vance et al. 1987). Soil respiration was
135 measured by the alkali trap method (Page et al. 1982). Soil dehydrogenase activity (DHA) was
136 measured using 2,3,5-triphenyltetrazolium chloride (3%) as the substrate (Casida et al. 1964).
137 The intensity of produced triphenyl formazan was measured colorimetrically at 485 nm using a
138 spectrophotometer (CE 2031, Cecil Instruments Ltd., Cambridge, UK). Alkaline
139 phosphomonoestarase (APM) (pH 11) activity in soil was determined as per described protocol
140 (Tabatabai & Bremner 1969). Soils were incubated in modified universal buffer (MUB) (2.42 g
141 tris-hydroxymethylaminomethane, 2.3 g maleic acid, 2.8 g citric acid and 1.26 g boric acid in 1 L
142 Milli-Q water, pH 11) using p-nitrophenyl phosphate as the substrate, and the produced yellow
143 color intensity of p-nitrophenol was measured at 440 nm on the above spectrophotometer. Soil
144 flourescein di-acetate (FDA) hydrolysis activity was assessed as described in (Adam & Duncan
145 2001) (Supplementary Information; SI3). Glomalin related soil protein (GRSP) content was
146 determined in rhizosphere soils (< 2 mm) using the established method (Wright & Upadhyaya
147 1998) (Supplementary Information; SI4).
148 The cultural diversity of soil beneficial microorganisms was determined by enumeration of the
149 total heterotrophic bacteria (nutrient agar medium), aerobic N- fixers (N-free Jensen's agar

150 medium) and P solubilizing bacteria (Pikovaskaya agar medium) using dilution plate techniques.
151 The colony forming units were expressed as log cfu g^{-1} soil.

152 **Statistical analysis**

153 Analysis of variance and pair wise test for cropping system with Bt and non Bt-cotton were
154 performed by fisher LSD test using XLSTAT software (Statistical software for Microsoft Excel
155 add on package). Duncan's multiple range test (DMRT) and LSD at $p < 0.05$ for comparison of
156 significant differences between means were performed using SPSS 20.0 (SPSS Inc., Chicago,
157 USA) package. Simple correlations were calculated between biological activities with the
158 various pools of soil organic carbon and carbohydrates to show their degree of associations.

159 **3. Results**

160 **Soil chemical and biochemical characteristics**

161 The soils were generally alkaline in reaction with pH values ranging from 7.1-7.4, and non-saline
162 (EC = 0.35-0.48 dS m^{-1}) in nature. The soils were low in available N (257- 293 mg kg^{-1}),
163 medium in available P (14.9- 19.0 mg kg^{-1}) and high in available K (184-221 mg kg^{-1}) contents
164 (values represent the average results obtained out of ten composite soil samples which were
165 taken from four different places randomly chosen). The water-soluble carbon (WSC) in soils
166 ranged from 9.4 to 15.6 mg kg^{-1}, and the mean values of acid-hydrolysable carbohydrate (AHC)
167 content ranged from 494 to 782 mg kg^{-1}. The average values of soil organic carbon (SOC) varied
168 from 4.8 to 7.7 g kg^{-1} in all the cotton based cropping systems. The farmers' fields adopted
169 recommended package of practices in the cotton-growing region. Significantly higher values ($p <$
170 0.05) of some of the chemical and biochemical parameters were noticed in the Bt-cotton based
171 cropping system compared to the non Bt-cotton based cropping system. The available nutrients

(N, P and K) showed slightly higher values under Bt-cotton than non Bt-cotton, but the difference was not significant ($p > 0.05$).

Soil enzymatic activities

Soil microbial parameters were studied by assessing soil enzymatic activities such as dehydrogenase (DHA), alkaline phosphomonoesterase (APM) and fluorescein di-acetate (FDA) hydrolysis activities (Figure 1, 2 and 3). Since the current experimental soils were neutral to slightly alkaline in reaction, only the APM activity was assessed. The APM enzyme prevails in alkaline soils (as in this study), whereas acid phosphomonoestarase generally dominates in acidic soils (Tabatabai & Bremner 1969). Higher values of DHA, APM and FDA hydrolysis activities were observed in the Bt-cotton soils compared to the non Bt-cotton soils (Figure 1, 2 and 3). Among different cropping systems, the highest soil DHA activity was observed in the Bt-cotton-soybean cropping system at all the developmental stages of cotton growth. A similar trend was followed in non Bt-cotton based cropping systems also.

Overall, the DHA (19.1 µg TPF g^{-1} soil h^{-1}), APM (243 µg PNP g^{-1} soil h^{-1}) and FDA hydrolysis (17 µg fluorescein g^{-1} soil) activities of soil were found significantly ($p < 0.05$) higher in the Bt-cotton-based cropping system than non-Bt systems (DHA, APM and FDA activities of 16.4 µg TPF g^{-1} soil h^{-1}, 214 µg PNP g^{-1} soil h^{-1} and 14 µg fluorescein g^{-1} soil, respectively) (Figure 1). Among the different stages of the crop growth, the boll development stage with Bt-cotton demonstrated a higher DHA, APM and FDA hydrolysis (22.7µg TPF g^{-1} soil h^{-1}, 253 µg PNP g^{-1} soil h^{-1} and 18 µg fluorescein g^{-1} soil, respectively) than the rest of the crop growth stages, e.g., vegetative stage and harvest stage.

Among various cropping systems, the cotton-soybean and cotton red-gram systems showed positive influence on soil enzyme activities than other cropping systems (Figure. 1, 2 & 3). The

activity of APM was also higher ($p < 0.05$) in the Bt-cotton based cropping system than in the non Bt-cotton system (Figure 2). The FDA hydrolysis activity was significantly higher ($p < 0.05$) in the cotton-soybean than in other cropping systems (Figure 3).

Soil microbial biomass carbon

Among different cotton based cropping systems, the cotton-soybean based system showed a significantly ($p < 0.05$) higher amount of soil microbial biomass carbon (MBC) than the other cropping systems. Overall, MBC of soil was found significantly ($p < 0.05$) higher in the Bt-cotton based cropping system (253 mg kg^{-1} soil) than the non Bt-cotton (218 mg kg^{-1} soil) based system (Figure 4). A higher soil MBC (270 mg kg^{-1} soil) was observed at the boll development stage of Bt-cotton than other crop growth stages.

Soil respiration

The highest soil respiration was observed in Bt-cotton-soybean cropping system at all the developmental stages of cotton. Non Bt-cotton based cropping systems also followed the same trend. Overall, the soil respiration was found significantly ($p < 0.05$) higher (16.8 mg CO_2-C kg^{-1} soil day^{-1}) in the Bt-cotton than non Bt-cotton (14.5 mg CO_2-C kg^{-1} soil day^{-1}) system (Figure 4).

Glomalin related soil protein (GRSP)

At all crop growth stages, the Bt-cotton based cropping system recorded a higher GRSP content (93-114 mg kg^{-1} soil) than non Bt-cotton system. Overall, the GRSP content of Bt-cotton soils (68 mg kg^{-1} soil) was significantly ($p < 0.05$) higher than non Bt-cotton soils (53 mg kg^{-1} soil) (Figure 5). The boll development stage of Bt-cotton recorded the highest GRSP content (mean of Bt-cotton based cropping system was 69 mg kg^{-1} soil).

Microbial population

The populations of viable soil microorganisms such as soil heterotrophs (5.7-7.9 log cfu g^{-1} soil), aerobic nitrogen fixers (3.9-5.4 log cfu g^{-1} soil) and P- solubilizers (2.5 -3.0 log cfu g^{-1} soil) were higher under Bt-cotton-soybean cropping system at all crop developmental stages than non Bt-cotton soils (Table 1). Average populations of soil heterotrophs (6.6 log cfu g^{-1} soil), aerobic nitrogen fixers (6.6 log cfu g^{-1} soil) and P- solubilizers (2.7 log cfu g^{-1} soil) were significantly ($p < 0.05$) higher in Bt-cotton based cropping systems than non Bt-cotton systems (Table 1). The boll development stage of Bt-cotton showed the maximum counts of different groups of soil microorganisms than the rest of the growth stages (Table 1).

Correlation studies

Results showed that among various C fractions, the active fractions of carbon (WSC and AHC) were the most sensitive indicators of soil quality in the current study (Table 2). There was a highly significant correlation between SOC and MBC ($r = 0.90$, $p < 0.01$), and between soil respiration and SOC ($r = 0.50$, $p < 0.01$). Similarly, significant correlation was observed between SOC and DHA ($r = 0.83$, $p < 0.01$), FDA ($r = 0.77$, $p < 0.01$), APM ($r = 0.75$, $p < 0.01$), AHC ($r = 0.48$, $p < 0.01$), WSC ($r = 0.55$, $p < 0.01$) and GRSP content ($r = 0.86$, $p < 0.01$). There was also a significant and positive correlation between AHC and MBC ($r = 0.60$, $p < 0.01$) and between WSC and MBC ($r = 0.61$, $p < 0.01$).

Discussion

Effect of Bt-cotton on soil biochemical properties

The rhizosphere of Bt-cotton showed higher values of all the carbon (WSC, AHC and SOC) fractions than that of the non Bt-cotton, which might be due to root exudates or low molecular weight organic compounds released in the rhizodeposits of Bt-cotton (Yan et al. 2007; Li et al. 2009), and have greater scope for future research. Not only the root exudates, but also a greater

biomass addition to the soil by Bt than non Bt-cotton is supposed to improve the C contents in the long-run. The higher concentrations of WSC and carbohydrates in the Bt-cotton than non Bt-cotton soils might also translate in to active pools of carbon that acted as the bio-energy for all microorganisms inhabiting the soil. The peak period of growth stages might have influenced the soil C pools under similar crop husbandry practices for both Bt and non Bt-cotton. Although the active pool is a small fraction of the SOM, its concentration is buffered by replenishment mechanisms such as desorption from soil colloids, dissolution from litter and exudation from plant roots (Six et al. 2000). The water-soluble fractions, including amino acids, organic acids and sugars, are considered the most active and highly labile fraction of carbon that is sensitive to intensive management practices. Secretion of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, and the secretions of proteins from Bt- and non Bt-cotton roots might have dissimilar effects on the inhabiting soil microorganisms (Chen et al. 2012). The available nutrient dynamics varied under Bt and non Bt-cotton might be due to the variation in their nutritional requirements and uptake by the existing crops (Sarkar et al. 2008).

Effect of Bt-cotton on soil enzymatic activities

The activity of DHA is considered as an indicator of the oxidative metabolism and microbiological activity in soils. Furthermore, carbon inputs from the plant rhizosphere influence the dynamics of microbial populations and their activity. Singh et al (2013a) reported that Bt-cotton grown in field conditions with combined application of urea and farm yard manure (FYM) maintained a higher soil DHA activity than other fertility treatments (individual N sources through urea and control without N). They also found that intercropping of Bt-cotton with peanut improved the DHA activity more than peanut as the sole crop. The rhizodeposits of transgenic cotton might have a greater impact than non-Bt cotton on the rhizospheric microorganisms and

263 enzymatic activities. A possible reason is that a greater rhizodeposition and addition of labile C
264 under Bt than non Bt-cotton might mask the negative impact of Bt-toxin (Singh et al. 2013a).
265 These changes might be transient depending upon the soil types, crop stages and environmental
266 conditions (Icoz & Stotzky 2008; Velmourougane & Sahu 2013). Some reports presented no
267 negative effect of cultivation of transgenic crops on soil enzymatic activities (Icoz et al. 2008; Li
268 et al. 2011). However, Chen et al (2012) reported an inhibitory effect of transgenic traits on the
269 activity of enzymes involved in nutrient cycling (C, N, P, and S). Lower enzymatic activities in
270 soil under the transgenic cotton were ascribed to the decrease in enzymes produced by soil
271 microorganisms, or to competition for the adsorption sites in soil among the Cry1Ac and CpTI
272 proteins and the enzymes (Sun et al. 2007).
273 The APM activity is associated with microorganisms that engage in soil P transformation. A
274 strong correlation was observed between APM activity and microbial biomass P under Bt-cotton
275 (Sarkar et al. 2008). The activities of β-glucosidase, nitrate reductase, phosphomonoesterase and
276 arylsulfatase were stimulated significantly in soils with Bt-cotton residue incorporation, but
277 DHA activity was suppressed due to the same (Chen et al. 2017).
278 Limited information is available on the effect of Bt-cotton on FDA hydrolysis in soil. In the
279 current study, the Bt-cotton soil showed a greater FDA hydrolysis than non Bt-cotton soil, which
280 was supported by Velmourougane & Sahu (2013). The higher values of FDA hydrolysis in Bt-
281 cotton soil also indicated a healthy microbial activity and no adverse effects of Bt-cotton on soil
282 microbial activities. However, the effects could vary under different soil types and agro-climatic
283 conditions (Chen et al. 2011). Soil types, clay and organic matter contents could influence the
284 degradation and binding of cry proteins in soils (Icoz & Stotzky 2008; Saxena et al. 2010).
285 **Effect of Bt-cotton on soil microbial biomass carbon and soil respiration**

286 Similar to the current study, a previous pot culture study also reported a significant improvement
287 of soil MBC due to Bt-cotton cultivation (Sarkar et al. 2009). Soil MBC might vary due to the
288 changes in weather conditions, type of crops and management inputs (Mandal et al. 2007). For
289 example, fertilization and manuring practices could change soil MBC (Luo et al. 2015). Chen et
290 al (2011) reported that soil MBC was inhibited by transgenic cotton proteins compared to their
291 non-transgenic controls. Similarly, Singh et al (2013b) reported that MBC was slightly reduced
292 in the transgenic brinjal soils, and the overall impact of transgenic brinjal was lower than non-
293 transgenic brinjal due to seasonal changes (Singh et al. 2013b). Contrarily, higher amounts of
294 MBC under various Bt-cotton and bulk soils were found in Indian Vertisols than non-Bt cotton
295 soils (Velmourougane & Sahu 2013). Therefore, seasonal changes along with soil types might
296 play an important role in influencing Bt-cotton's effect on soil MBC. Similar to soil MBC, soil
297 respiration was also found the least at the vegetative stage (30-45 days after sowing, DAS), and
298 the highest at the boll development stage (100-120 DAS). The improvement in soil organic
299 matter and microbial quotient (MBC to TOC ratio) in Bt–cotton soil might have played direct
300 roles in enhancing the soil respiration (Sarkar et al. 2009; Yasin et al. 2016). In the present study,
301 the labile C fraction did not improve the soil C status due to its low chemical stability, but long-
302 term Bt-cotton cultivation over years may impart positive impact on SOC buildup.

303 **Effect of Bt-cotton on glomalin related soil protein (GRSP)**

304 GRSPs are hydrophobic glycoproteins that play important role in soil organic carbon persistence
305 and sequestration (Singh et al. 2017). GRSP could accumulate up to several g kg^{-1} and might
306 account for 52% of total C in an organic soil (Gao et al. 2017). The amount of GRSP could be
307 representative of the presence and activity of arbuscular mycorrhizal (AM) fungi in the rhizosphere.
308 Information on mycorrhizal colonization on Bt-cotton roots is limited in the literature. A very few
309 reports are available where AM fungi were preferentially studied as an indicator for ecological

impacts of genetically modified plants on soil microbial communities (Tan et al. 2011). In the present study, a higher amount of GRSP observed at the boll developmental stage of Bt-cotton-soybean cropping system might be due to an increased nutrient availability, which would have a favourable effect on the colonization of the fungi. Increased fungal population in Bt-cotton soil (Xie et al. 2016), actinomycetes population in transgenic brinjal soil (Singh et al. 2013c) and AM colonization under legume-based system (Nijra et al. 2017) support the present findings.

Effect of Bt-cotton on soil microbial population

Soil microorganisms play critical roles in a variety of biological functions in both the rhizosphere and the soil near decomposing plant residues. Plant residues are the primary source of metabolic energy (carbon) in soils, and the majority of biotic populations are concentrated in the rhizosphere. Therefore, any change in the quality of crop residues and rhizosphere inputs could potentially modify the microbial dynamics. Zhang et al (2016) reported no significant difference in the population size of major soil microbial groups between transgenic and non-transgenic wheat rhizospheres, and changes in the population counts were attributed to growth stages of the crop. Variation in the population of soil microorganisms in Bt and non-Bt rhizospheres found in this study was likely due to differential levels of root exudates quantity, composition and root characteristics of the transgenic cotton (Yan et al. 2007; Kapur et al. 2010).

Implication in farmers' fields

In the cotton belts of Central India, Maharashtra, farmers usually do not apply adequate quantity of organic manures to soils due to lack of availability of inputs at right time. Comparatively reduced microbial count in soils under farmers' cultivation than the research experimental farm (Table 3) could be due to the subtle effect of organic manure application on soil microorganisms and plants in cotton fallow system at the two locations (research farm and farmers' field). The nutrient management practices for both Bt and non Bt-cotton were similar though in the

experimental fields and adjoining farmers' plots. Addition of organic manure in the farmers' fields could improve the soil microbiological attributes and offset adverse effect of Bt-toxin released in the rhizosphere, if any (Hu et al. 2011; Singh et al. 2013a). However, similar to Li et al. (2011) the current study did not indicate any reduction of microbial activity or deterioration of soil health by the cultivation of transgenic Bt-cotton per se.

Conclusion

This study revealed that soil biochemical and microbiological activities under Bt-cotton based cropping system was significantly different from non Bt-cotton based cropping system. Among the cropping systems, the cotton-soybean and cotton-red gram systems showed higher values for the biochemical parameters than cotton-wheat, cotton-vegetables and cotton-fallow systems. Greater microbial activities and biochemical properties were observed in the Bt-cotton than non Bt-cotton soils that could be attributed to a substantial enhancement in the soluble phase of organic C originating from rhizodeposition, root biomass and leaf-litter, which would act as a source of bio-energy for soil microorganisms. However, Bt-cotton cultivation in experimental plots or farmers' fields in this study indicated no significant depletion of soil microbial activity or selected functional microbial populations. Future research should attempt to measure soil Bt-toxin levels under field conditions and correlate them with soil biochemical parameters and microbial communities at molecular scale.

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List of figure captions

Figure 1. Effect of different cotton-based cropping systems on dehydrogenase activities at (A) vegetative stage, (B) boll development stage and (C) harvest stage. Histograms with different small case letters are statistically significant at p = 0.05. Error bars represent ± standard errors.

Figure 2. Effect of different cotton-based cropping systems on alkaline phosphomonoesterase activities in soil at (A) vegetative stage, (B) boll development stage and (C) harvest stage. Histograms with different small case letters are statistically significant at p = 0.05. Error bars represent ± standard errors.

Figure 3. Effect of different cotton-based cropping systems on fluorescein diacetate hydrolysis activities at (A) vegetative stage, (B) boll development stage and (C) harvest stage. Histograms with different small case letters are statistically significant at p = 0.05. Error bars represent ± standard errors.

Figure 4. Effect of different cotton-based cropping systems on soil microbial biomass carbon and soil respiration at (A) vegetative stage, (B) boll development stage and (C) harvest stage. Histograms with different small case letters are statistically significant at p = 0.05. Error bars represents ± standard errors.

Figure 5. Effect of different cotton-based cropping systems on glomalin related soil protein (GRSP) contents at (A) vegetative stage, (B) boll development stage and (C) harvest stage. Histograms with different small case letters are statistically significant at p = 0.05. Error bars represent ± standard errors.

Figures

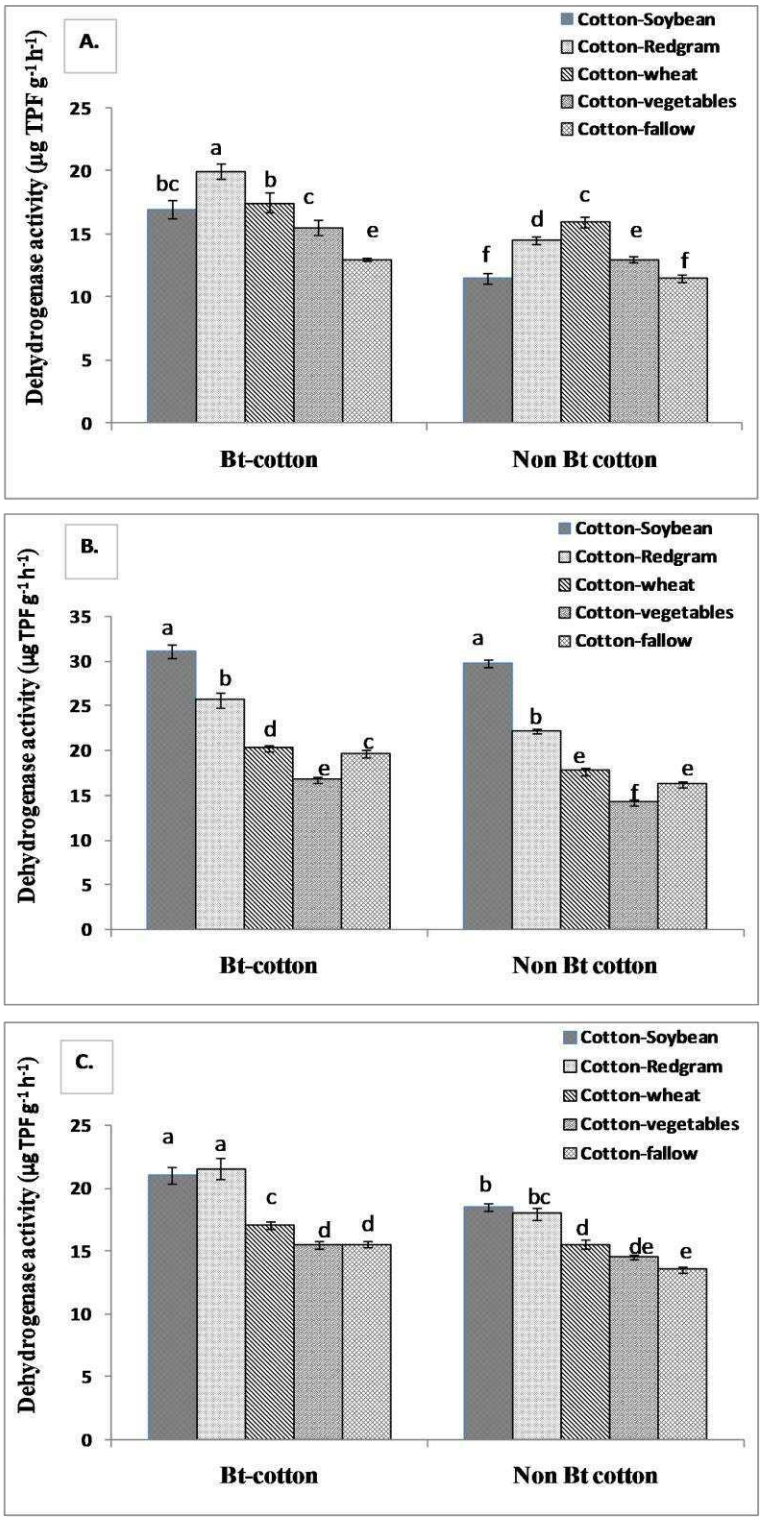
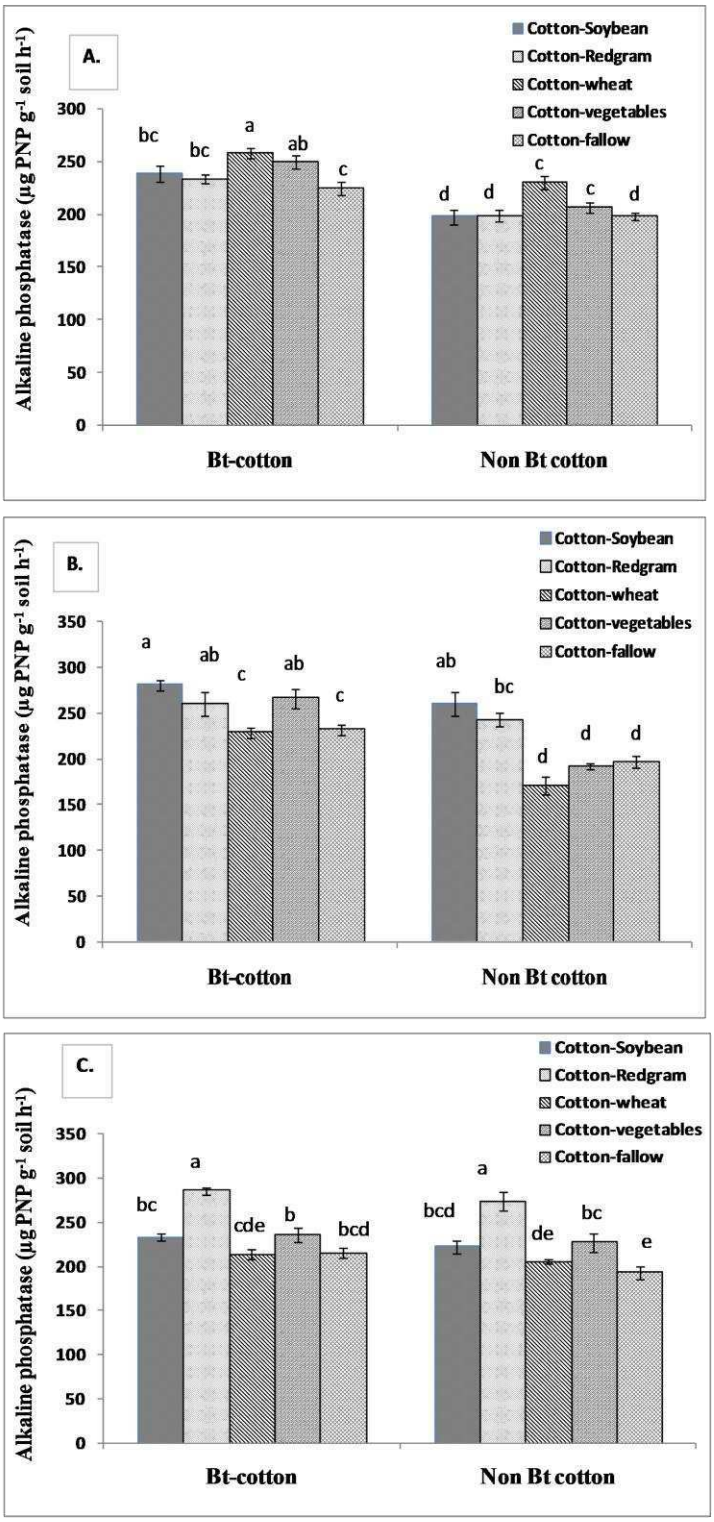


Fig. 1



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506 Fig. 2

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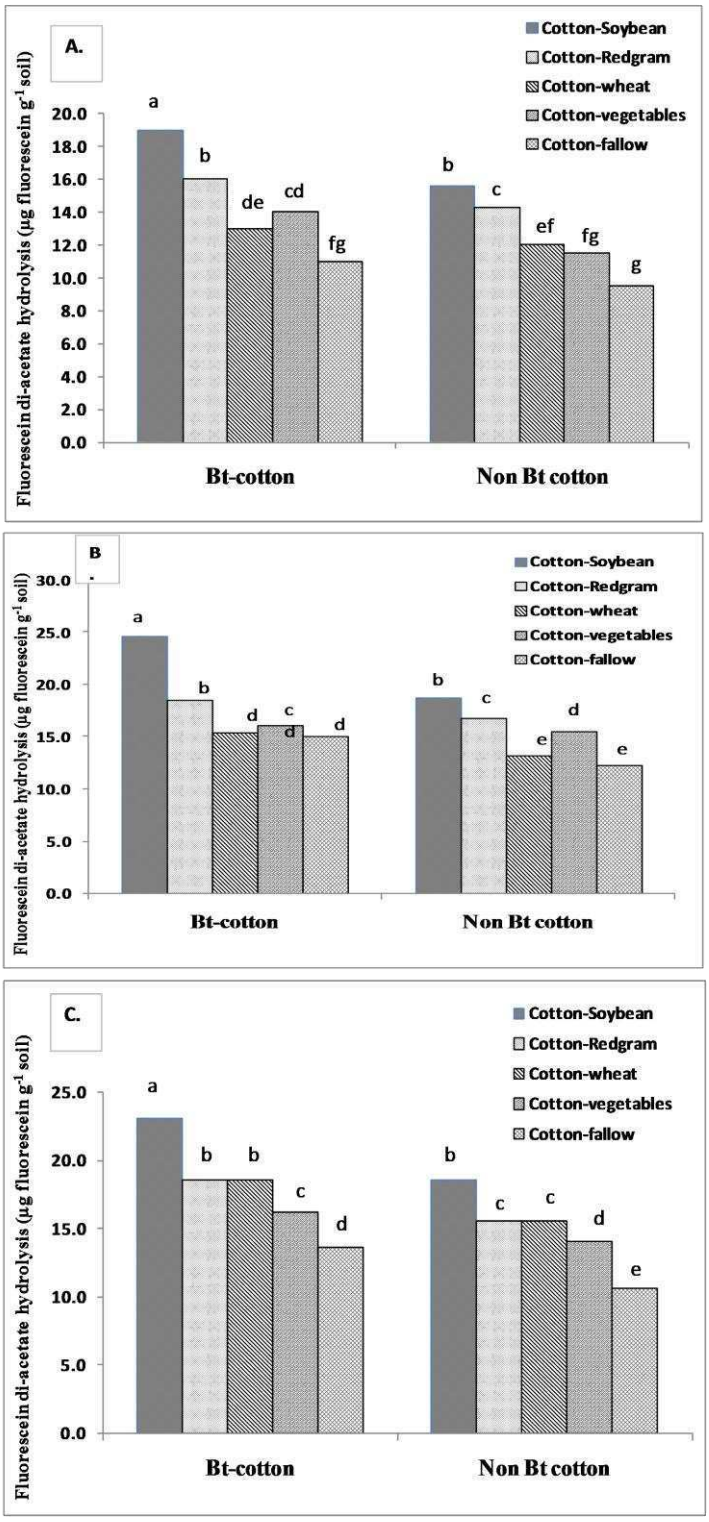

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509 Fig. 3

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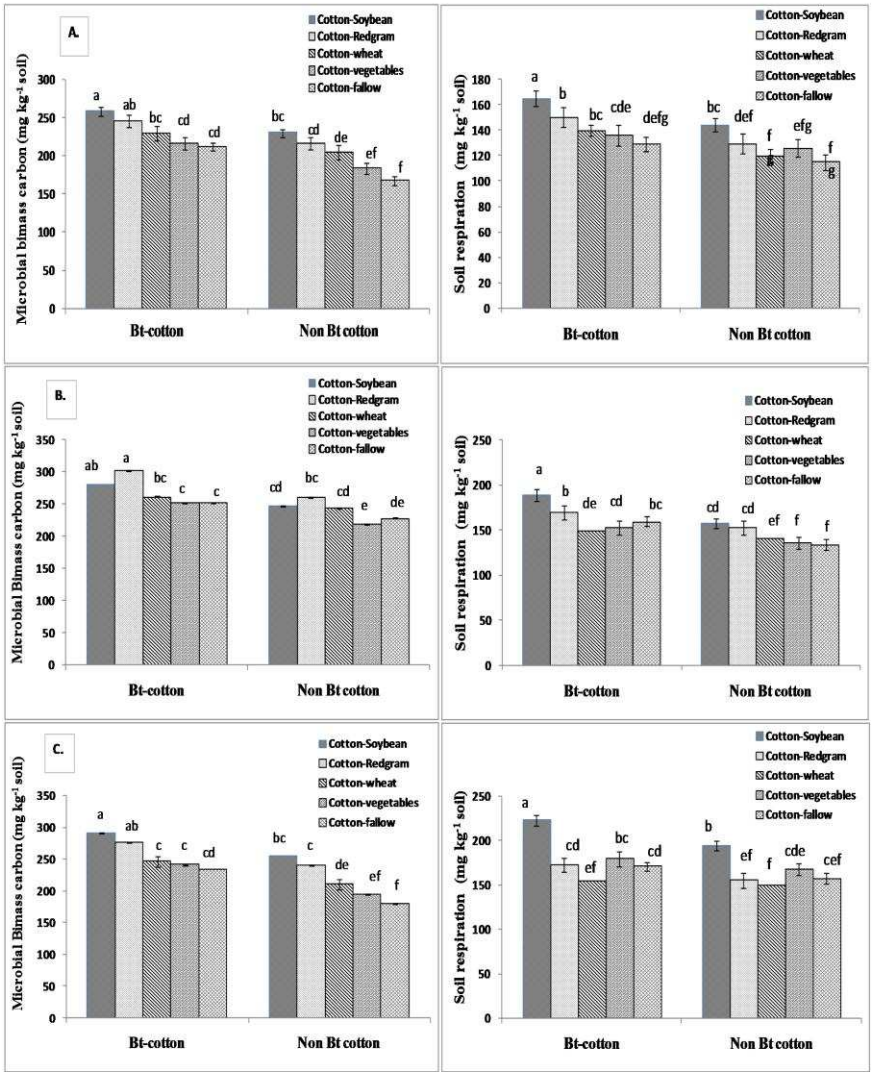

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512 Fig. 4

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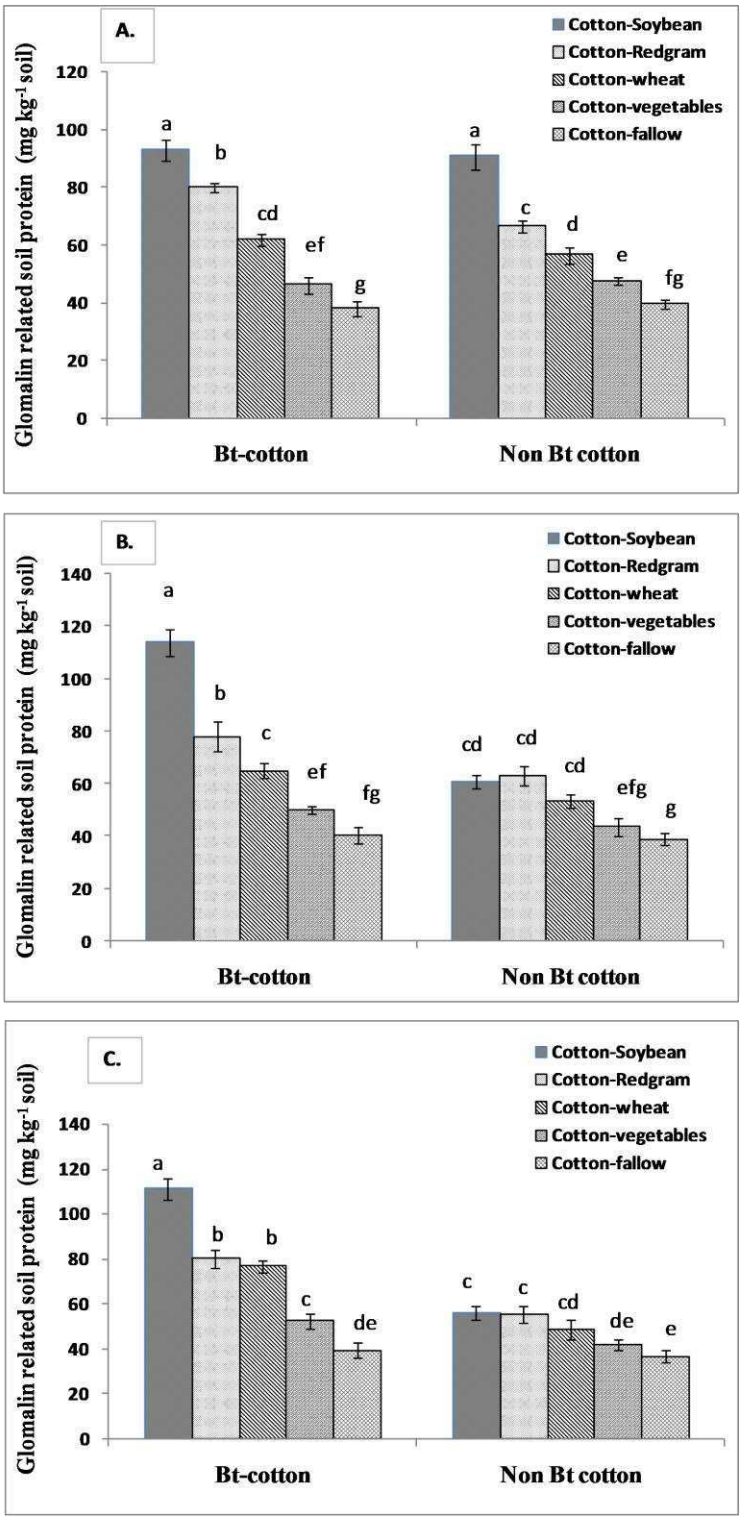
A.
Cotton-Soybean
Cotton-Redgram
Cotton-wheat
Cotton-vegetables
Cotton-fallow
Glomalin related soil protein (mg kg-1 soil)
120
100
80
60
40
20
0
a
b
cd
ef
g
a
c
d
e
fg
Bt-cotton
Non Bt cotton
B.
Cotton-Soybean
Cotton-Redgram
Cotton-wheat
Cotton-vegetables
Cotton-fallow
Glomalin related soil protein (mg kg-1 soil)
140
120
100
80
60
40
20
0
a
b
c
ef
fg
cd
cd
cd
efg
g
Bt-cotton
Non Bt cotton
C.
Cotton-Soybean
Cotton-Redgram
Cotton-wheat
Cotton-vegetables
Cotton-fallow
Glomalin related soil protein (mg kg-1 soil)
140
120
100
80
60
40
20
0
a
b
b
c
de
c
c
cd
de
e
Bt-cotton
Non Bt cotton

514

515 Fig. 5

516 **Table 1.** Effect of cropping systems and different growth stages of transgenic Bt-cotton on soil heterotrophic bacterial population, aerobic

517 nitrogen fixers population and phosphate solubilizer population (log cfu g^{-1} soil)

Soil heterotroph population (log cfu g^{-1} soil)						
	Vegetative stage		**Boll development stage**		**Harvest stage**	
Cropping system	**Bt-cotton**	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**
Cotton-soybean	6.4a¶ ±0.23	5.2b±0.15	8.3a±0.17	5.9de±0.25	5.4bcd±0.31	5.0de±0.21
Cotton-redgram	6.6a±0.31	5.2b±0.06	8.1ab±0.26	6.1d±0.21	6.3a±0.24	5.1cde±0.15
Cotton-wheat	6.4a±0.23	6.0ab±0.32	7.6bc±0.20	5.6de±0.12	5.8abc±0.26	5.9ab±0.29
Cotton-Vegetables	5.6ab±0.42	5.9ab±0.31	7.3c±0.25	5.4e±0.23	5.2bcde±0.23	4.9de±0.23
Cotton-fallow	6.3a±0.21	4.1c±0.21	5.4e±0.23	5.4e±0.35	6.2a±0.23	4.6e±0.26
Aerobic N-fixers (log cfu g^{-1} soil)						
Cotton-soybean	4.1a¶ ±0.21	3.9a±0.12	5.0c±0.06	5.2bc±0.15	3.7a±0.29	3.4ab±0.15
Cotton-redgram	4.1a±0.25	3.8ab±0.23	5.5ab±0.21	5.4bc±0.17	3.8a±0.15	3.5ab±0.15
Cotton-wheat	4.1a±0.21	3.8ab±0.10	5.9a±0.12	5.1bc±0.12	3.6ab±0.21	3.4ab±0.15
Cotton-Vegetables	3.7abc±0.12	3.2c±0.10	5.4bc±0.21	5.2bc±0.15	3.4ab±0.17	3.1bc±0.15
Cotton-fallow	3.6abc±0.12	3.3c±0.06	5.2bc±0.23	5.2bc±0.15	3.3abc±0.21	2.8c±0.17
P-solubilizers (log cfu g^{-1} soil)						
Cotton-soybean	3.0a¶ ±0.15	2.7ab±0.12	3.7a±0.25	3.2ab±0.15	3.0a±0.21	2.7abc±0.15

Cotton-redgram	2.5ab±0.23	2.6ab±0.15	3.0ab±0.15	2.9ab±0.21	2.9ab±0.15	2.7abc±0.15
Cotton-wheat	2.5ab±0.12	2.4b±0.15	3.0ab±0.12	2.7ab±0.23	2.9ab±0.15	2.6abc±0.12
Cotton-vegetables	2.3b±0.12	2.5ab±0.15	2.6b±0.21	2.5b±0.12	2.5bc±0.21	2.3c±0.15
Cotton-fallow	2.2b±0.15	2.4b±0.17	2.4b±0.13	3.4ab±0.15	2.4c±0.21	2.4c±0.15

Data represent mean values (n = 3) ± their standard error. ¶Mean data points with different lower case letters within a row and column for a particular measurement is significantly different according to Duncan's Multiple Range Test (DMRT) at $p < 0.05$. The data for different growth stages were analyzed separately.

523 **Table 2.** Pearson's correlation (r) matrix for soil biochemical and enzymatic activities (overall values under Bt- and non Bt crops)

524 during cotton growth

Properties	WSC[§]	AHC	SOC	SMBC	SR	DHA	FDA	APM	GRSP
WSC	1	0.34*	0.55**	0.61**	0.40**	0.36*	0.47**	0.50**	0.47**
AHC		1	0.48**	0.60**	NS	0.48**	0.54**	0.55**	0.65**
SOC			1	0.90**	0.50**	0.83**	0.77**	0.75**	0.86**
SMBC				1	0.48**	0.83**	0.76**	0.81**	0.86**
SR					1	0.58**	0.76**	0.64**	0.43**
DHA						1	0.76**	0.78**	0.71**
FDA							1	0.77**	0.80**
APM								1	0.73**
GRSP									1

525

526 [§]WSC: water soluble carbohydrate, AHC: acid hydrolysable carbohydrate, SOC: soil organic carbon, SMBC: soil microbial biomass

527 carbon, SR: soil respiration, DHA: dehydrogenase activity, FDA: fluorescein di-acetate activity, APM: alkaline phosphomonoestarase

528 activity, GRSP: glomalin related soil protein. *p = 0.05, **p = 0.01 significant correlations.

529

530 **Table 3.** Comparison of soil microbial cultural diversity data between CICR experimental farm and farmers' fields grown with

531 transgenic Bt-cotton

	Vegetative stage		**Boll development stage**		**Harvest stage**	
	Soil heterotrophs (log cfu g^{-1} soil)					
	Bt-cotton	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**
CICR farm	6.3±0.36*	4.1±0.36	5.4±0.40	5.4±0.60	6.2±0.40	4.6±0.46
Farmers field	5.4±0.32	4.0±0.15	5.1±0.31	4.9±0.53	5.1±0.25	4.2±0.38
	Aerobic N-fixers (log cfu g^{-1} soil)					
	Bt-cotton	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**
CICR farm	3.6±0.20	3.3c±0.10	5.2±0.40	5.2±0.26	3.3±0.36	2.8±0.30
Farmers field	3.2±0.21	3.0 ±0.35	4.2±0.49	4.1±0.25	3.2±0.44	2.7±0.46
	P-solubilizers (log cfu g^{-1} soil)					
	Bt-cotton	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**	**Bt-cotton**	**Non Bt-cotton**
CICR farm	2.2±0.26	2.4±0.30	2.4±0.23	3.4±1.79	2.4±0.36	2.4±0.26
Farmers field	2.0±0.21	1.8±0.35	2.2±0.49	2.3±0.25	2.1±0.44	2.0±0.46

532

533 *Data represent mean values (n = 3) ± their standard deviations.

534 Supplementary Information for:

535 **Effects of Bt-cotton on biological properties of Vertisols in central India**

536

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549

SI1. Determination of acid-hydrolysable carbohydrate (AHC)

Acid-hydrolysable carbohydrate (AHC) was determined by method (Brink 1960). In brief, 5 g air dried soil samples (passed through 1-mm sieve) placed in a steam bath (85^{o}C throughout the hydrolysis reaction time of 24 h) with 50 mL of 3N H_2SO_4 in a placed in 125 mL Erlenmeyer flask covered with a glass lid to minimize evaporation. The hydrolyzate was then passed through sintered G-4 filter of medium pore size and the residue was washed with 50 mL of hot water (85^{o}C). Anthrone (0.2%) was made up in 95% sulfuric acid at least an hour before use. Appropriately diluted soil hydrolyzate (5 mL) was pipetted into a test tube, and shaken well. The colorimetric readings of samples were taken on a spectrophotometer (CE 2031, Cecil Instruments Ltd., Cambridge, UK) at 625 nm against a water-anthrone blank.

SI2. Determination of water-soluble carbon (WSC)

The water-soluble carbon (WSC) was determined as per the outlined procedure (McGill et al. 1986). In brief, WSC was extracted from field-moist soils (10 g) within 24 h of sampling by shaking with 20 mL deionizer water for 60 min, followed by centrifugation at l0,000 x g for 30 min. The supernatant was further filtered upon suction through a 0.2-pm metricel membrane filter (47 mm diameter) which was previously washed with 150 mL deionized water. The filtrates were stored at –l0^{o}C until analyzed. Carbon in the filtered aliquot was digested in a mixture of 0.07N $K_2Cr_2O_7$ (5 mL), concentrated H_2SO_4 (10 mL) and ortho-phosphoric acid (5 mL). The sample was mixed carefully, and digested at 150^{o}C for 30 min using a digestion block, and cooled to room temperature. Thereafter, 1 mL of diphenylamine indicator was added, and titrated against 0.035N ferrous ammonium sulphate prepared in 0.4M H_2SO_4.

SI3. Analysis of flourescein di-acetate (FDA) hydrolysis activity

574 Soil flourescein di-acetate (FDA) hydrolysis activity was assessed by the method of Adam and
575 Duncan (2001).In brief, 2 g of soil (fresh weight, 2-mm sieved) was incubated for 30 min with
576 the substrate FDA (0.2 mL of 2000 µg mL^{-1} solution) in 15 mL of potassium phosphate buffer
577 (pH 7.6). The produced fluorescent color (extracted with 15 mL of chloroform/methanol, 2:1
578 v/v) was measured using the same spectrophotometer stated above (490 nm) following
579 centrifuging the aliquot at 2000 x g for 3 min.
580
581 SI4. Analysis of Glomalin related soil protein (GRSP)
582 Glomalin related soil protein (GRSP) content was determined in rhizosphere soils (< 2 mm)
583 using the method of Wright and Upadhyaya (1998). Easily extractable GRSP was solubilized in
584 20 mM citrate buffer at pH 7 by autoclaving at 121ºC for 30 min, and the total GRSP was
585 extracted in 50 mM citrate buffer (pH 8) by autoclaving for 90 min. For the sequential
586 extractions, the supernatant was removed by centrifugation at 5000 x g for 20 min. Extraction of
587 samples was continued until the supernatant showed no red brown colour typical of glomalin.
588 Extracts from each replicate were pooled, and analysed. After extraction cycles were completed,
589 samples were further centrifuged at 10,000 x g to remove soil particles, and protein in the
590 supernatant was determined by the Bradford method with bovine serum albumin as the standard
591 (Wright and Upadhyaya 1998).
592
593 **References**
594

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