Impact of transgenic cotton varieties on activity of enzymes in their rhizosphere

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The impact of five Bacillus thuringiensis (Bt) cotton varieties and their respective isogenic non-Bt (NBt) isolines (ANKUR-2534, MECH-6304, RCH-317, ANKUR-651 and MECH-6301) was assessed on the key soil enzymes i.e., dehydrogenase, alkaline phosphatase and urease in their rhizosphere at four growth stages of the crop, namely vegetative, flowering, bolling and harvesting. These varieties were grown on farmer’s field in villages 22 miles and 24 miles of Ganganagar District of Rajasthan State in India. Results showed that dehydrogenase, alkaline phosphatase and urease activities were higher in rhizosphere of Bt isolines as compared to NBt isolines of all the varieties. Except phosphatase, differences in dehydrogenase and urease activities in rhizosphere of Bt and NBt isolines of all five varieties were significant (P<0.05). Maximum enhancement in the three enzymes activities was observed in MECH-6304 Bt isolate rhizosphere. Maximum and minimum activities of dehydrogenase and urease were observed in MECH-6304 and RCH-317 Bt isolines, respectively, whereas phosphatase activity was maximum and minimum in MECH-6304 and ANKUR-651 Bt isolines, respectively. Maximum dehydrogenase and urease activities were observed at boll formation and minimum at flowering and harvesting stage, respectively, while maximum phosphatase activity was observed at vegetative stage and minimum at harvesting stage. In conclusion, all the studied Bt isolines of cotton varieties showed no adverse effect on dehydrogenase, alkaline phosphatase and urease activities in the rhizosphere.

Keywords: Bt-Cotton, Dehydrogenase, Urease, Phosphatase, Soil enzymes, Growth stage

India is one of the major cotton producers in the world having the largest acreage under cotton and is also the second largest consumer of cotton. Bt-transgenic cotton was introduced in India in 2002. Following its success during the last 9 years (2002-2011), the area under Bt-cotton has increased by 8.4 mha from 0.029 mha1. A total of 522 Bt-cotton varieties were approved for planting in 2009 compared with 274 Bt-cotton varieties in 2008, 131 in 2007, 62 in 2006, 20 in 2005 and only 4 Bt-cotton varieties in 20041.

Although there are diverse benefits of Bt-cotton varieties, public concern also exists. While Bt bacteria occur naturally in soil, growth of cropping area under Bt-crops causes a large increase in the amount of Cry endotoxin present in agricultural systems, e.g. roughly 0.25 g ha⁻¹ produced naturally (calculated from approx 1000 Bt spores g⁻¹ soil vs. 650 g ha⁻¹ in case of Bt-corn crop, excluding grain)². Both in vitro and in vivo studies on Bt-cotton have shown that Bt-toxin produced in its leaves, stems and roots is finally introduced in soil by two major pathways-biomass incorporation and root exudates3-5.

In soil, released Bt-toxin is adsorbed on clay particles, humic components, or organic mineral complexes and protected against degradation by soil microorganisms⁶. Thus, the Bt-cotton, either through the Bt-toxin and modified rhizosphere chemistry or through altered crop residue quality has the potential to significantly change the soil ecosystem functions⁷-¹¹ and the soil biochemical properties⁹,¹²-¹⁵. As the rhizosphere (the zone directly surrounding and influenced by plant roots) contains a majority of soil microbial biota, the plant-microbe interaction in rhizosphere is one of the major factors regulating nutrient transformation and hence also the health and growth of plants. Therefore, any change in the quality of root exudates in rhizosphere can modify the biota composition in the soil (biodiversity) as well as their functions⁸.

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Several experiments have been conducted to assess risks from Bt-cotton on flora and fauna in diverse agroecosystems\textsuperscript{15-19}. Results of some experiments have shown no negative effects of Bt-cotton on soil flora and fauna and may even have beneficial effects\textsuperscript{3,14}, while some have reported adverse effects\textsuperscript{21,22,36}. However, such experimental studies on risk assessment of Bt-cotton with respect to soil ecosystem in India are very limited. Climatically, India is a subtropical country, thus as compared to temperate and sub-temperate countries, biological and biochemical response of Indian soil to continuously increasing acreage of Bt-cotton may vary.

The soil enzymes dehydrogenase, phosphatase and urease are part of some of the important soil biochemical processes and through them play an essential role in energy transfer, organic matter decomposition, nutrient cycling and crop productivity\textsuperscript{24,27}. Dehydrogenase has been widely used to measure catabolic activities in soil, which is correlated with microbial activity\textsuperscript{30}. Urease is responsible for the breakdown of urea into carbon dioxide and ammonia and owing to this property, has an applied importance in the N-economy of soil. Phosphatase mediates the release of inorganic phosphorus from organically bound phosphorus returned to soil as litter and other organic debris and affects the rate of phosphorus cycling.

We hypothesize that Bt-cotton varieties may adversely affect soil enzymes activity. Therefore, in this study, impact of 5 Bt-cotton varieties, namely ANKUR-2534, MECH-6304, RCH317 ANKUR-651 and MECH-6301 (approved by Genetic Engineering Approval Committee for commercial cultivation in Northern India) and their respective isogenic non-Bt (NBt) i.e., non-transgenic cotton varieties grown on farmers’ field on enzymes dehydrogenase, phosphatase and urease has been monitored at different growth stages during crop growth period.

### Materials and Methods

#### Study site

The sites for the present study were farmer’s field located in villages-22 miles and 24 miles of Ganganagar district of Rajasthan State, India. Three varieties, namely ANKUR-2534, MECH-6304, RCH-317 Bt and NBt isolines and other two varieties, namely ANKUR-651 and MECH-6301 Bt and NBt isolines were grown in farmers field of villages 22 miles and 24 miles, respectively. MECH, RCH and ANKUR Bt-cotton hybrid varieties monitored in the study had Cry1AcBt gene for insect pests resistance and were developed by Indian seed companies, namely-MAHYCO (Maharashtra Hybrid Seed Co.), Rasi Seeds and Ankur Seeds, respectively under license agreement with the US seed multinational, Monsanto. Soils of sites were sandy loam, alkaline and had low cation-exchange capacity (CEC) (Table 1). Seeds of the selected Bt and NBt isolines were sown after treating with fungicide thiram and imidacloroprid (Bayer). During the cotton crop growth (May-October) period, average rainfall and temperature of the area were 250-300 mm and 35-37°C, respectively.

#### Soil sampling

Rhizospheric soil samples of the Bt and NBt isolines of five varieties were collected at regular interval (30 days interval, coinciding with different growth stage of cotton) since the date of sowings till harvesting. First sampling was done 60 days after sowing (DAS) i.e., at vegetative stage (growth stage 1), second sampling 90 DAS i.e., at flowering stage (growth stage 2), third sampling on 120 DAS i.e., at cotton boll formation stage (growth stage 3) and fourth sampling was 150 DAS i.e., at mature stage (growth stage 4). Ten random rhizosphere samples (0-15 cm depth) were collected using auger for each Bt and NBt isolines for analysis. Collected rhizospheric soil samples were processed by air drying, grounding and sieving (passed through a 2 mm sieve) homogenized and stored in the refrigerator at 4°C and analyzed within 3-4 days. For observing dehydrogenase activity, the soil samples were kept moist.

### Dehydrogenase activity

Dehydrogenase activity was estimated as described previously\textsuperscript{25}. 5 g of soil was incubated for 12 h at 37°C in 5 ml of a triphenyl tetrazolium chloride

### Table 1—Properties of soil at study sites in Ganganagar District of Rajasthan

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Village-22 ML</th>
<th>Village-24 ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (1: 2.5 soil to water)</td>
<td>8.45</td>
<td>8.25</td>
</tr>
<tr>
<td>Electrical conductivity</td>
<td>0.38</td>
<td>0.28</td>
</tr>
<tr>
<td>(1: 2.5) dS m(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>0.32</td>
<td>0.47</td>
</tr>
<tr>
<td>Phosphorus (kg/ha)</td>
<td>30.00</td>
<td>22.50</td>
</tr>
<tr>
<td>Potash (kg/ha)</td>
<td>445.00</td>
<td>460.00</td>
</tr>
<tr>
<td>Zn (ppm)</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td>Cu (ppm)</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Fe (ppm)</td>
<td>1.12</td>
<td>3.08</td>
</tr>
<tr>
<td>Mn (ppm)</td>
<td>1.15</td>
<td>1.79</td>
</tr>
</tbody>
</table>
(TTC) solution (5 g TTC in 0.2 M Tris–HCl buffer, pH 7.4). Two drops of conc. H2SO4 were added immediately after the incubation to end the reaction. The sample was then blended with 5 ml of toluene and shaken for 30 min at 250 rpm, followed by centrifuging at 4500 g for 5 min to extract triphenyl formazon (TPF). The optical density of the red colour supernatant was measured spectrophotometrically at 492 nm using UV–Vis spectrophotometer (UV-1201, Shimadzu Corp, Japan). Soil dehydrogenase activity was expressed as µg TPF g−1 12 h−1

Alkaline phosphatase activity
Alkaline phosphatase activity was measured spectrophotometrically as described previously24. One g soil was placed in a 50 ml Erlenmeyer flask and treated with 0.25 ml of toluene and 4 ml modified universal buffer (MUB) (prepared by dissolving 12.1 g Tris, 11.6 g of maleic acid, 14 g citric acid and 6.3 g boric acid in 500 ml 1 M NaOH and diluted the solution to 1000 ml with distilled water) of pH 11 and 1 ml of p-nitrophenyl phosphate solution (2.927 g disodium p-nitrophenyl phosphate in about 40 ml MUB and bring volume upto 50 ml with the buffer). Thereafter, the flask contents were mixed and incubated for 1 h at 37°C. After 1 h of incubation, 1 ml of CaCl2 (0.5 M) and 4 ml of NaOH (0.5 M) were added to the flask. The colored soil suspension was filtered through Whatman grade no. 2 filter paper and absorbance of filtrate was measured at 400 nm. The phosphatase activity was expressed as µg p-nitrophenol g−1 h−1

Urease activity
Urease activity in soil sample was estimated according to “determination of urea remaining” methodology25. This methodology estimates urea hydrolysis in soils on account of urease activity. For this, 5 g of soil mixed with 5 ml of urea solution (0.01 g urea/ml) in a 50-ml Erlenmeyer flask and incubated for 5 h at 37°C. After 5 h, 50 ml 2 M KCl-phenyl mercuric acetate (PMA) was added to flasks and kept for 1 h shaking. Thereafter, soil suspension was filtered under suction through Whatman no. 42 filter paper. Out of filtrate 2 ml of aliquot was taken and mixed with 10 ml 2M KC1-PMA and 30 ml coloring reagent (25 ml 2.5% diacetylmonoxime (DAM) + 10 ml of 0.25% thiosemicarbazide (TSC) in 500 ml acid reagent). This mixture was first kept in water bath for 30 min and then kept in ice cold water for 15 min for color development. Absorbance of colored end product was measured at 527 nm. Activity of urease in soil was expressed as µg urea N hydrolyzed g−1 h−1

Experimental design and statistical analysis
This study was designed as a randomized complete block. Main block treatments were 5 different varieties and their respective Bt and NBt isolines. The Bt and NBt isolines (considered as treatments) of each variety and growth stages during crop growth period were considered as source of variation. The interactions between treatments (Trt), cotton varieties (Var) and growth stage (grstg) were used for testing significance (P-value based) of the sources of variation. The statistical analysis of the experimental data was done using JMP 8 statistical software.

Results
Average activity of dehydrogenase, phosphatase and urease was high in rhizosphere of Bt isolines as compared to NBt isolines. Except phosphatase, differences in dehydrogenase and urease activities in rhizospheres of Bt and NBt isolines of all the five varieties were statistically significant (P<0.05) (Table 2). Over the crop growth period, mean activity of dehydrogenase and phosphatase in the rhizosphere of five varieties Bt and NBt isolines was maximum at vegetative stage, whereas urease activity was maximum at boll formation stage (Table 3). Activity of all the enzymes declined at crop harvesting stage (Table 3).

Effects of varieties (var), growth stages (grstg), treatments (trt) as well as interactive effects of varieties and growth stages (vr*grstg), varieties, growth stages and treatments (var*grstg*trt) on dehydrogenase activity in rhizosphere soil samples of all the five varieties were significant (P<0.05) (Table 2). Whereas interactive effects of varieties and treatments (var*trt), growth stages and treatments (grstg*trt) on dehydrogenase activity in rhizospheric soil of all the five varieties were non significant (P<0.05) (Table 2). Dehydrogenase activity was higher in rhizosphere of Bt isolines, as compared to NBt isolines of all the five varieties (Fig. 1). Mean dehydrogenase activity was maximum in MECH-6304 and minimum in RCH-317 rhizosphere. Maximum dehydrogenase activity was observed at growth stage 3 (at time of boll formation) and minimum at growth stage 4 (at maturity). At growth stage 4, significant difference (P<0.05) in dehydrogenase activity in rhizosphere of Bt and NBt
isolines of MECH-6301, RCH-317 and ANKUR-651 was observed. At growth stages 1, 2 and 3, these differences in rhizosphere of Bt and NBt isolines of MECH-6301 and RCH-317 were non-significant. Dehydrogenase activity in rhizosphere of Bt and NBt isolines of ANKUR-651 was significantly different at growth stages 1, 2 and 4. In MECH-6304 and ANKUR-2534, dehydrogenase activity in
rhizosphere of *Bt* and NBt isolines was significantly different only at growth stages 3 and 2, respectively (Table 3).

Urease average activity was higher in *Bt* isolines as compared to NBt isolines of all the varieties, except MECH-6304 (Fig. 1) and was maximum in rhizosphere of RCH-317 and minimum in MECH-6304. Maximum urease activity was observed at growth stage 3 i.e. 120 DAS and minimum at growth stage 2. Urease activity was significantly affected by individual and interactive effects of cotton varieties (Var), *Bt* and NBt isolines (trt) and growth stages (grstg) (Table 2). At growth stage 1, no significant difference in activity in rhizosphere of *Bt* and NBt isolines of all five varieties was observed. At growth stage 2, significant difference in activity in rhizosphere of *Bt* and NBt isolines of all varieties, except RCH-317 was observed. At growth stage 3, activity in rhizosphere of *Bt* and NBt isolines of MECH-6304, MECH-6301 and ANKUR-651 was significantly different, whereas in RCH-317 and ANKUR-2534 varieties it was non-significant. At growth stage 4, urease activity in rhizosphere of *Bt* and NBt isolines of MECH-6304, MECH-6301 and ANKUR-2534 was significantly different, whereas in RCH-317 and ANKUR-651, it was non-significant (Table 3).

Variation in phosphatase activity in rhizosphere of *Bt* and NBt isolines was statistically non-significant (P>0.05). Variation in phosphatase activity due to individual effect of variety and growth stage and interactive effect of variety, growth stage and treatment was significant (Table 2). Average phosphatase activity was higher in *Bt* isolines as compared to NBt isolines of all the varieties, except MECH-6301 (Fig. 1). Mean phosphatase activity was maximum and minimum in rhizosphere of *Bt* isolate of MECH-6304 and ANKUR-651, respectively. Maximum phosphatase activity was observed at growth stage 1 and minimum at growth stage 4. At growth stage 1, difference in phosphatase activity in rhizosphere of *Bt* and NBt isolines was significant only for MECH-6304, MECH-6301 and ANKUR-2534. At growth stage 2, significant difference in phosphatase activity in rhizosphere of *Bt* and NBt isolines of ANKUR-651 and ANKUR-2534 varieties was observed. At growth stage 3, phosphatase activity was significantly different only in rhizosphere of *Bt* and NBt isolines of RCH-317. At growth stage 4, phosphatase activity in rhizosphere of *Bt* and NBt isolines of MECH-6304, MECH-6301, RCH-317 and ANKUR-651 was significantly different, whereas in ANKUR-2534 it was non-significant (Table 3).

**Discussion**

Soil microorganisms are one of the main sources of soil enzymes, which play an essential role in energy transfer, environmental quality, organic matter decomposition, nutrient cycling and crop productivity. Dehydrogenase activity is only present in viable cells is a useful indicator of overall microbial activity in soil. Phosphatases are believed to play critical role in phosphorus cycles by catalyzing hydrolysis of esters and anhydrides of phosphoric acid. Urease plays a vital role in the hydrolysis of urea fertilizer and nitrogen availability to plants. Therefore, activities of soil enzymes act as key biological indicator of changes occurring in soil.

In the present study, enhancement in enzymes activities in rhizosphere of five *Bt* isolines was observed as compared to their respective NBT
Bt-cotton (MECH-6301) and its NBt hybrid have significant differences in root volume between is directly related to microbial activity. Previously, root volume and biomass of plants at this stage, which were reported to be large at flowering stage might be because of large urease in NBt isolines of all the varieties. Stimulation in urease, phosphatase, invertase and cellulase activities in soil by the addition of Guo Kang 12 and Zhong-Kang 30 Bt-cotton tissues is reported. In present study, variations in dehydrogenase and urease activities in rhizosphere of all five varieties, due to Bt and NBt isolines were statistically significant (P<0.05). Earlier, enhanced dehydrogenase activity also has been reported in soil incubated with Bt transgenic rice straw (KMD line) (during 7-14 days of incubation, later on it declined), compared to soil without straw. However, no significant differences in dehydrogenase activity in Compa Cb and MON 810 Bt-maize varieties and KMD 1, Bt rice variety rhizosphere, respectively have been observed.

The enhancement in enzymes activities in rhizosphere of Bt isolines of all varieties might be due to increased as well as altered composition of root exudates. Enhanced exudation of amino acids and sugars release from roots of transgenic cotton varieties (Zhong-41 and GK-12) as compared to its parental non transgenic line (Zhong-23 and SM-3) has been reported. Root exudates have a profound qualitative and quantitative effect on the rhizospheric microbial activity and plant growth. In conclusion, five varieties of Bt cotton monitored in present study did not have any adverse effect on dehydrogenase, alkaline phosphatase and urease activities in their rhizosphere. Enhanced activity of soil enzymes in ANKUR-2534, MECH-6304, RCH-317 ANKUR-651 and MECH-6301 varieties rhizosphere due to variety as well as growth stages overweighed the Bt and NBt isolines. However, since the results reported were based on farmer’s field experiments where cultivation practices were not 100% controlled, there is a need to confirm these findings in controlled field and lab studies.

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