Spanish Journal of Agricultural Research
17 (2), e0303, 8 pages (2019)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2019172-14605
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A, M.P. (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Effects of *Cry* toxins on non-target soil bacteria during a 2-year follow up study

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Abstract

Genetically modified (GM) plants with insecticidal *Bacillus thuringiensis* (Bt) genes are widely accepted but their commercial utilization highlights the biosafety issues worldwide. The risk assessment of GM crops demonstrates their impact on the ecosystem as well as non-target organisms (NTOs). Among the NTOs, plant growth promoting rhizobacteria (PGPR) demand more critical experimental studies as they play a significant role in plant growth. A comparative study of Bt with non-Bt cotton rhizosphere was conducted, on selected bacterial strains. During the course of the study, biochemical characterization, auxin biosynthesis and molecular characterization was done to assess the effect of Bt toxins (*Cry1Ac* and *Cry2A*) on non-target PGPR strains. A significant decrease ($p<0.05$) in phosphatase activity was recorded in some of the experimental bacterial strains as compared to those of control strains. However, no significant differences ($p>0.05$) were observed in other parameters like bacterial population, colony morphologies as well as biochemical activities. Thus, our study demonstrates the safe plantation of Bt crops with respect to soil bacteria.

Additional keywords: Bt crops; risk assessment; *Cry* toxins; soil; bacteria.

Abbreviations used: Bt (*Bacillus thuringiensis*); CFU (colony forming unit); GM (genetically modified); MS (maturity-setting stage); NTOs (non-target organisms); SS (seeding stage).

Authors' contributions: Conceived, designed and performed the experiments: AY and AAS. Analysed the data: IBS. Contributed reagents/materials/analysis tools: SA, AQR and MA. Wrote the paper: AY and IBS. Rephrased the final manuscript: AQR. All authors read and approved the final manuscript.

Citation: Yaqoob, A.; Shahid, A. A.; Salisu, I. B.; Azam, S.; Ahmed, M.; Rao, A. Q. (2019). Effects of *Cry* toxins on non-target soil bacteria during a 2-year follow-up study. Spanish Journal of Agricultural Research, Volume 17, Issue 2, e0303. https://doi.org/10.5424/sjar/2019172-14605

Received: 28 Jan 2019. **Accepted:** 05 Jul 2019.

Funding: University of the Punjab, Centre of Excellence in Molecular Biology, Pakistan.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

The development of genetically modified (GM) crops is a big bang in the history of agriculture which transformed the conventional agricultural system into an advanced one using genetic tools and molecular approaches (Udriste & Badulescu, 2017). We have witnessed a revolution in traditional agriculture just after the successful advent of transgenic varieties in late 1970s. One of the leading GM traits is insect resistance, where *Bacillus thuringiensis* (Bt) genes transformed into the plant, express a toxic protein (*Cry*) which targets the gut epithelium of attacking insect and eventually protects the host plant from pathogens (Lee *et al.*, 2016). Transgenic crops with Bt insecticidal gene can specifically protect itself from Lepidopterans, *i.e.* cotton bollworm, corn borer, tobacco beetle, pink bollworm and budworms (Dutton *et al.*, 2002). The economical benefits of Bt plantation include the decreased pesticidal sprays and increased crop productivity. The global hectarage of Bt crop cultivation is reported to be greatly increased from 1.68 million hectares in 1995 to 181.5 million hectares in 2014 (Liang *et al.*, 2018).

Despite the popularity of Bt crops, their regular cultivation, however, could result in unintentional effects on soil organisms. In view of this, a number of risk assessment studies have been conducted in order to evaluate the potential unintended effect of Bt crops on non-target organisms (NTOs). However, most of the outcomes of such studies were controversial (Tsatsakis *et al.*, 2017). As such, the need for further safety assessment of Bt crops on NTOs, particularly the beneficial soil microbes cannot be ignored because Bt

toxin, when expressed by plant cells, could influence the soil organisms by entering the soil either through plant residues or via root exudates (Strain & Lydy, 2015). But, contrarily, further studies ruled out this threat by reporting a rapid degradation of Bt protein soon after its release into the soil (Li *et al.*, 2016). Although it was declared that Bt protein does not persist for long term in soil, the risks are still associated with their short-term existence in rhizosphere, as the Bt proteins or even genetic material can possibly be taken up by soil microbes through natural phenomena of transformation. Hence, a critical investigation is needed to assess the potential impacts of Bt crop rhizosphere on soil bacteria specially the plant growth promoting rhizobacteria (PGPR) which are the most important and least studied group of microbiotas (Yaqoob *et al.*, 2016).

PGPR are significant elements of plant rhizosphere, promoting plant growth and improving the soil structure through aggregate formation, nitrogen fixation, nitrate/nitrite reduction, mineral solubilization, increasing the organic contents of rhizosphere and phytohormone production (Yaqoob *et al.*, 2013). Among PGPR, *Pseudomonas* and *Bacillus* sp. are widely distributed in soil but least studied for GM risk assessment. Therefore, these bacterial rods were selected specifically for the present study to assess the potential risks of Bt toxin on these soil bacteria.

Material and methods

Ethics declaration

No private or protected land was used or disturbed in any way. Protected or endangered organisms were not involved in any experimentation.

Cotton cultivars

We used transgenic Bt cotton (*Gossypium hirsutum*) cv. VH-289 (provided by CEMB repository) transformed with *Cry1AC* and *Cry2A* genes expressed under 35S constitutive promoter using *Agrobacterium*-mediated transformation. The transgenic plants possess the appropriate concentrations of Cry/Bt toxin in their leaves, stem, boll, roots and other parts. Non-transgenic cotton variety was used as negative control.

Field setup and sampling

A randomized block design (31°33'N, 74°19'E) was set out for two consecutive growing seasons (2017–2018) of cotton. The transgenic lines (VH-289) were grown under appropriate field conditions for cotton growth. Agronomic practices such as fertilization, watering, hoeing and weeding were done under the standard conventional method. The physical and chemical properties of the soil were determined (Table 1). Fifteen experimental plants of *G. hirsutum* were selected both for root and soil sample collection (each in triplicate) at two developmental stages: seed-setting (SS) and maturity-setting (MS) stages. The average rainfall and temperature were also estimated during both SS and MS stages (Table 2). All samples were collected from the soil rhizosphere under sterile conditions by digging the soil up to 7-9 cm near roots (for SS stage) and by separating soil clumps adhered to roots during harvesting (for MS stage) as shown in Fig. 1. The collected samples were then sieved through an aperture size of 830 µm and stored at 4ºC immediately.

Root analysis for *Cry* proteins

Roots of experimental and control plants were dug out at plant harvesting time, carefully washed, cut into 2 cm fragments, and sterilized with 10% KOH for 20 min. Root extracts were obtained by two ways: (1) grinding of roots in protein extraction buffer (Dikova, 2011) and (2) soaking the root pieces in water for 60 days. Detection of *Cry1AC* and *Cry2A* proteins was done using commercially available ELISA kit based on highly specific purified anti-*Cry1AC* and *Cry2A* rabbit polyclonal antibody assay. The data were validated using the guidelines of Envirologix kit (catalog # AP003).

Isolation of soil bacteria

Serial dilutions of soil samples (0.1 µg/sample) were made. Fifty microliters of the soil samples (diluted

Table 1. Electrical conductivity (EC), pH, total organic contents, phosphorus, potassium, saturation and texture of rhizosphere soil.

Treatments		EC (mS/cm)	P (mg/kg)	K (mg/kg)	Saturation (%)	pH	Texture
2017	SS	2.803±0.015	8.66±0.011	186±1.0	35±1.0	7.7±0.15	Loam
	MS	2.79±0.015	7.8±0.03	175±1.0	37.3±1.5	7.1±0.15	Loam
2018	SS	3.03±0.015	7.7±0.2	166±1.0	30±1.0	6.9±0.15	Clay/Loam
	MS	2.80±0.09	7.8±0.2	170±1.0	32±1.0	7.2±0.25	Clay/Loam

SS: seedling stage; MS: maturity-setting stage.

Table 2. Mean monthly temperature and rainfall during the experiment.

	June (SS)	September (MS)
2017		
Temperature (°C)	34.2	24.5
Rainfall (mm)	91.7	56.3
2018		
Temperature (°C)	35.0	23.3
Rainfall (mm)	92.9	48.78

SS: seedling stage; MS: maturity-setting stage.

up to 10^{-3}) was inoculated on soil extract agar plates (Hamaki *et al.*, 2005) and incubated at 37ºC for 24 hours. Number of viable bacterial cells was counted by conventional plate count/colony forming unit (CFU) method: CFU/mL = (No. of colonies × dilution factor)/ volume (mL) of culture plate. Plate counter was used to count colonies. Plate counter contains a light source and magnifying glass making colonies visible (Dutton *et al.*, 2002). Bacterial colonies were then subjected to gram's staining (Prophet *et al.*, 1992) and observed under the compound microscope using oil immersion lens. The gram-negative and gram-positive rods were isolated on L-agar plates.

Biochemical characterization

Biochemical tests such as nitrate reduction (Gamble *et al.*, 1977), nitrogen fixation (Jensen, 1951) and phosphate solubilization were performed on isolates from both experimental and control plant groups. Phosphate solubilization was checked by an agar assay using the National Botanical Research Institute's phosphate (NBRIP) medium (Islam *et al.*, 2007). The index of solubilization was measured: Phosphate solubilization index = Colony diameter/Total diameter of clear zone around the colony.

The overnight bacterial cultures were inoculated to L-Broth supplemented with 2% L-tryptophan and incubated at 37ºC for 72 hours in the shaker. Twenty microliters of bacterial culture was transferred to a separate vial and centrifuged at 3000 rpm for 5 min. The supernatant was taken and its pH was maintained at 2.5 (acidic). One ml of supernatant was mixed with 2 mL of Salkowski's reagent and test tubes were left in the dark for 30 min to develop a red color. The intensity of color was measured at 535 nm. A standard curve for indole acetic acid was drawn by using different concentrations of authentic auxin to determine auxin biosynthesis in the bacterial culture supernatant (Yaqoob *et al.*, 2013).

Statistical analysis

The data presented in tables and figures are means with standard deviation (SD). All data were analyzed using analysis of variance to find any differences between the control mean and that of the experimental groups. Graph-pad prism (Version 5.0) for Windows was used for all the analyses.

Molecular characterization of PGPR strains

The overnight bacterial culture (1.5 mL) was centrifuged at 13000 rpm for 5 min, followed by the addition of 400 µL of CTAB buffer (0.5 M EDTA, 5 M NaCl, 1 M Tris-HCl, 10% CTAB, 10% β-mercaptoethanol, 5% proteinase K solution) to the pellet. Afterward, 150 µL of Solution II (10 mM NaOH and 10% SDS) was added and mixed gently by resuspension. The mixture was incubated at 60ºC for 2 hours followed by addition of 500 µL PCI solution (phenol: chloroform: isoamylalcohol). The mixture was centrifuged for 10 min at 13000 rpm and aqueous phase (supernatant) was carefully transferred to a freshly

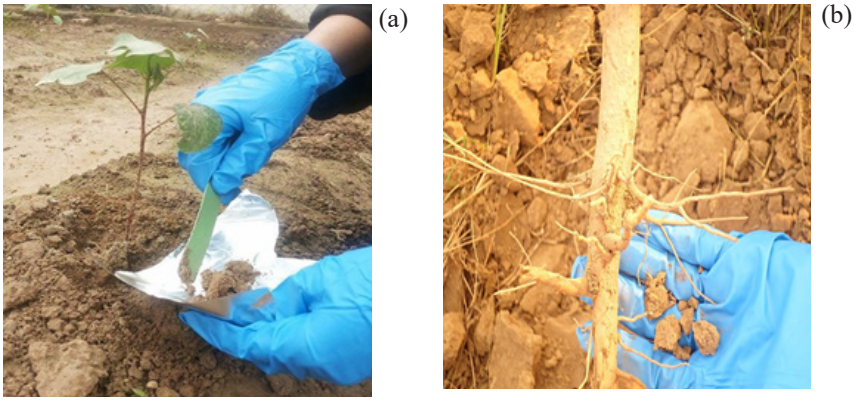

Figure 1. Sample collection from rhizosphere of transgenic cotton variety (a) at seedling stage (SS, June) by digging the soil up to 10 cm; (b) at harvesting time (MS, September) by digging out the root

labeled tube and 300 µL of isopropanol (100%) was added to it. A final incubation of 10 min was given at room temperature followed by 20 min at -20ºC, centrifuged for 5 min at 13000 rpm and supernatant was discarded. The DNA pellet was washed with 1 mL of 70% ethanol and then air dried. DNA pellet was finally resuspended in 30 µL of deionized distilled water.

The experimental and control strains which showed promising results for biochemical characterization were selected for the 16S ribotyping. 16S rDNA fragments were amplified by PCR using universal primers; RS-F 5'-AAACTCAAATGAATTGACGG-3' and RS-R 5'-ACGGGCGGTGTGTA-3'. PCR products were sequenced with a SequiTherm cycle sequencing kit (ABI 3730 genetic analyzer, Applied Biosystems).

Results

Proteins *Cry1AC* and *Cry2A* were detected in all the collected soil samples. However, a significant decrease in concentration of *Cry* proteins was noticed when root pieces were soaked in water as compared with direct protein extraction method (Fig. 2).

Fig. 3 shows the bacterial colony count from both control and experimental groups during the two years. There were no significant differences (p>0.05) in bacterial count at both experimental periods.

More than 90% of all the strains were found to be positive for nitrate reduction and nitrogen fixation tests. No noticeable difference was observed between the control and experimental strains by comparing their test results. However, significant decrease (p<0.05) in phosphate solubilization ability was observed in experimental strains at maturity stages when compared with control (Fig. 4).

Significant potential for auxin biosynthesis was revealed by all bacterial strains upon the stimulation of L-tryptophan and a reduced auxin production was observed in experimental strains at maturity stages when compared with that of control isolates (Fig. 5).

The 16S rDNA sequences of experimental strains 03-N4, 03-N9, 01-I and 08-H were deposited in the NCBI under accession nos. JQ912576.1, AB920753.1, KP698780.1, and KF732997.1 respectively, and sequences of control strains 04-P5 and 06-N5 under no. JN861778.1 and KP685409.1, respectively.

Analysis of a 450-650 bp sequence of 16S rDNA from six representative bacterial strains indicated that three strains (03-N9, 03-N4 and 04-P5) exhibited ≥90% similarity to *Pseudomonas* spp., while the other three (01-1, 08-H and 06-N5) exhibited ≥90% similarity to *Bacillus* spp. (Table 3).

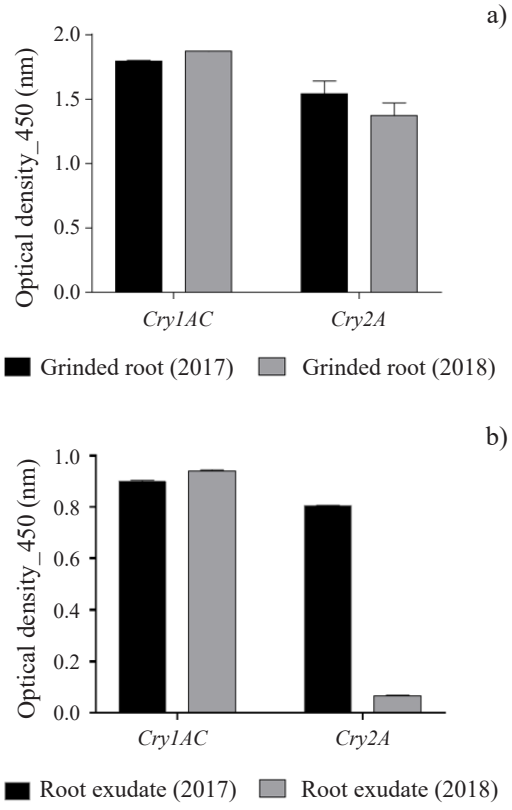

Figure 2. Relative concentration (µg) of *Cry1AC* and *Cry2A* proteins detected in root samples among two years of plantation (2017-2018).

Discussion

Soil is the basic medium of growth for all plants. The soil ecosystem plays a significant role in food chain, housing not only the macroorganisms but also the plant growth promoting microorganisms. Besides this ecological role, the contribution of soil components in nutrient cycling is also worth mentioning: a primary role in nutrient cycling is played by soil microbes due to their diverse ability of solubilizing complex organic/inorganic compounds into simpler molecules or ions. These molecules or ions when dissolved in soil particles become easily available to plant roots for their proper growth and metabolism (Prasad *et al.*, 2015). Therefore, plant rhizosphere is considered to be most important for risk assessment studies against Bt crop cultivation. The present study was planned to specifically assess the potential effects of Bt Cotton (VH-289) on soil bacterial count during two consecutive years (2017–2018). It was observed that both plant growth stage and planting date impacted soil microflora but the Bt trait was the weediest clarifying factor to impart any significant change. Our results were in agreement with those of

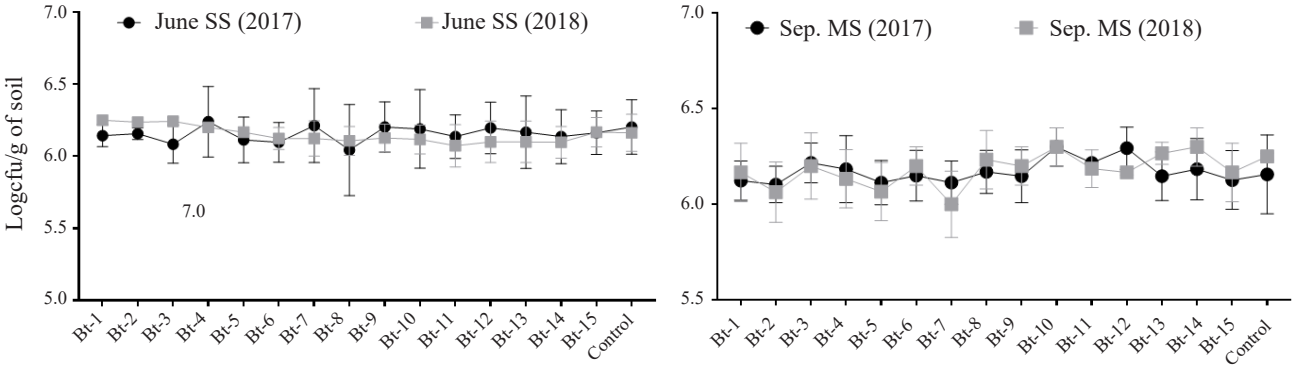

Figure 3. Bacterial colonies count in fifteen experimental samples in each set. All the values in the figure are means ± SEM. Two-way analysis of variance showed non-significant differences (p>0.05) between all groups from 2017 and 2018.

Liang *et al.* (2015), who found that cultivation of GM plants did not affect the soil microbial community structure.

Along with the biosafety assessment, flow rate of *Cry* proteins from roots into the external medium was also assessed to confirm previous contradictory studies on the release of Bt protein into the soil. For this purpose, both experimental and control plant roots were soaked in distilled water for two months and the water was then used for protein extraction and *Cry1AC*/*Cry2A* estimation through ELISA. It was revealed that very minute quantities of *Cry* proteins were released into the water as compared to total extracted protein (as shown in Fig. 5). Our findings are in accordance with Yaqoob *et al.* (2016), who found a decreased persistence of Bt proteins in soil.

The biochemical activities of the bacterial isolates were found to be non-significantly affected. However, a reduced rate of phosphate solubilization and auxin biosynthesis was observed during maturity stages of

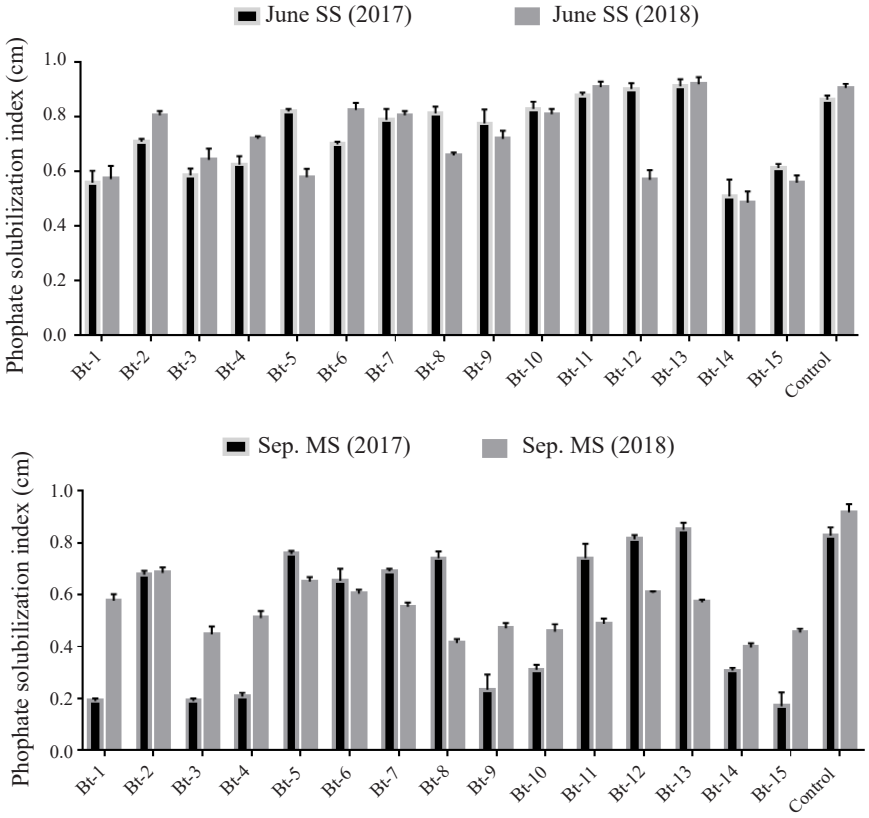

Figure 4. Phosphate solubilization assay of bacterial isolates from experimental soil samples (means ± SEM). Two-way analysis of variance indicated no significant differences (p>0.05) between most of the groups from 2017 and 2018.

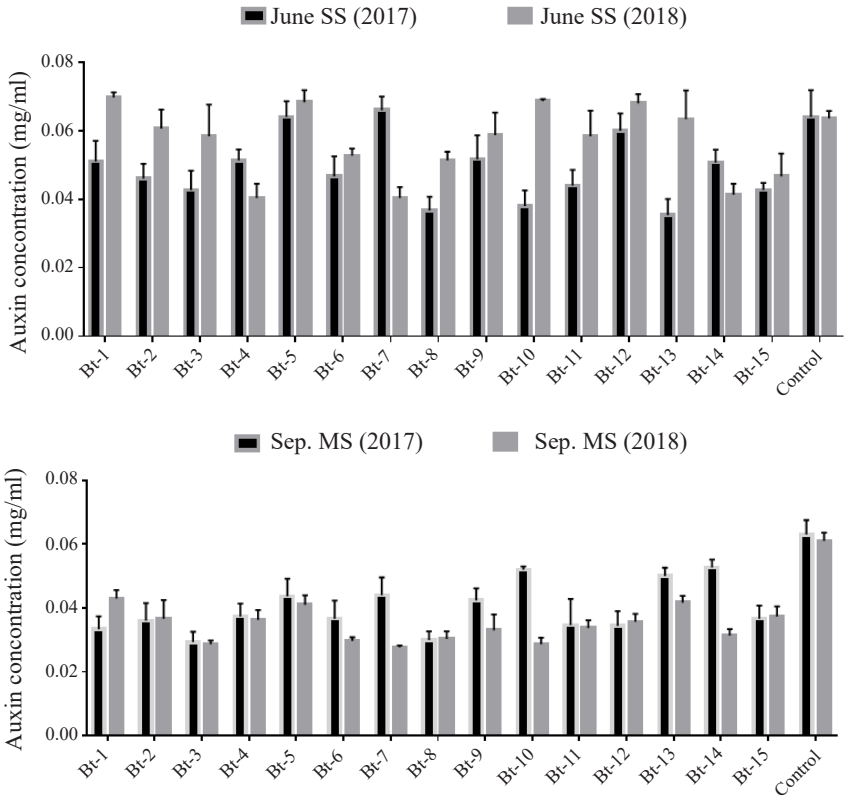

Figure 5. Auxin biosynthesis assay of bacterial isolates from experimental soil samples (means ± SEM). Two-way analysis of variance indicated no significant differences (p>0.05) in most groups from 2017 and 2018.

both years when compared with that of early stages. Potentially this reduced auxin biosynthesis might be occurred as per the need of plant growth, *i.e.* more auxin is produced at early growth stages of plants for excessive root and shoot growth as compared to maturity stages. Similarly, environmental factors and weather conditions must also be kept in consideration while assessing the bacterial activities.

The impact of transgenic plants on bacterial communities in soil could be either temporary or have no effect at all (Rashid *et al.*, 2016). According to Hannula *et al.* (2012) and Liang *et al.* (2018) minor changes in microbial populations that occur due to transgenic plants are mostly due to change in season and field and these changes do not persist in next field season. The findings of this research is in line with Li & Liu (2013), Wu *et al.* (2014) and Zhang *et al.* (2015), who found that Bt toxins released from transgenic plants had no short-span lethal effects on soil bacterial populations, but conclude that long-term impact due to toxin accumulation on biodiversity of soil needs to be evaluated.

Differences of temperature, rainfall and soil characteristics were observed in both planting years with

Table 3. Homology (%) of isolated strains with other microbes.

Bacterial isolate	Acc. No.	Fragment length (bp)	Nearest homology (Acc. No.)	Homology (%)
Transgenic lines				
03-N9	AB920753.1	471	*Pseudomonas aeruginosa* (PA157)	97
03-N4	JQ912576.1	477	*Pseudomonas* spp. (DL5.7)	99
01-I	KP698780.1	470	*Bacillus cereus* (RMRCBF6)	98
08-H	KF732997.1	489	*Bacillus mycoides* (PMM13)	98
Control lines				
04-P5	JN861778.1	532	*Pseudomonas aeruginosa* (BS PGPR)	90
06-N5	KP685409.1	660	*Bacillus mycoides* (LAR 1)	90

significant differences in total soil pH ($p<0.01$). These differences could also be the reason of effects on biochemical activities of bacteria as rhizobacterial activities are moderately affected by change in soil pH (Simonin *et al.*, 2016). Different soil properties and other environmental parameters in different seasons significantly affect soil microbial population.

The present study concluded that presence of minute quantities of Bt toxin in soil rhizosphere is non-toxic to soil bacteria. No harmful effect is noticed on biochemical or molecular characteristics of isolated soil bacteria from *Bt* cotton rhizosphere except a decreased phosphatase activity in some of the strains. The information presented in this study is useful for Bt crop development and its biosafety. However, more critical investigation is required to assess the risks of Bt crops on other soil micro and macrorganisms.

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