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COMPARISON OF RHIZOSPHERIC BACTERIAL POPULATIONS AND GROWTH PROMOTION OF *AVP1* TRANSGENIC AND NON-TRANSGENIC COTTON BY BACTERIAL INOCULATIONS

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ABSTRACT

Transgenic plants with desirable gene are biotechnological tool to alleviate food deficiencies in sustainable agriculture. Cotton (*Gossypium hirsutum* L.) is an important fiber crop and a source of raw materials for the textile industry. Drought stress is an utmost factor that ravages cotton quality and limits crop production. Transgenic plants with overexpression of *AVP1* gene have ability to survive in salt and water stress conditions. However, introduction of transgenic plants can affect rhizosphere environment. Bacterial abundance, survival and plant growth promotion activities can be good indicators to study the effects of transgenic plants in rhizospheric environment. Present study demonstrated that culturable bacterial populations were not significantly different in the rhizosphere of *AVP1* transgenic (TC) and non-transgenic (NTC) cotton plants at three different growth stages. Three bacterial strains were isolated from the rhizosphere of cotton and identified as *Arthrobacter*, *Azospirillumand Brevibacillus* spp. based on 16S rRNA gene sequence analysis. Plant growth promoting traits i.e. phosphate solubilization and phytohormone (IAA) production were detected in all three bacterial isolates. Growth promotion of transgenic and non-transgenic cotton plants by inoculation of these three strains indicated significant increase in growth and yield of both TC and NTC plants. Maximum growth promotion was observed in plants inoculated with *Brevibacillus* spp. strain TN4-3NF which showed an increase of 17.9% in root dry weight, 18.4% in shoot dry weight, 17.6% in yield of TC-pants, and 14.4% in root dry weight, 5.5% in shoot dry weight, and 13.9% in yield of NTC-plants compared with respective non-inoculated controls. From the present study it can be concluded that transgenic plants have no apparent drastic effect on rhizosphere microflora and both the *AVP1* transgenic and non-transgenic cotton plants are equally responsive to bacterial inoculations.

Key words: Transgenic plants, rhizosphere bacterial population, 16S rRNA gene, PGPR, phytohormone IAA, phosphate solubilization, plant inoculation.

INTRODUCTION

Cotton (*Gossypiumhirsutum* L.) is an important source of raw materials for the textile industry and the backbone of national economies of cotton growing countries. Among different approaches being used to combat water shortage and quality problems, the development of transgenic plantsis more feasible and directed approach for drought and salinity tolerance but risk assessment of these plants is indispensable for sustainable ecosystem functioning (Li *et al.,* 2010).There is tremendous increase in the adoptability and cultivation of genetically modified crops in recent years (Gruissem, 2015). In 2014, 28 countries grew GM crops and the global hectarage of biotech crops has increased more than 100-fold from 1.7 million hectares (1996) to 181.5 million hectares (2014). Pakistan ranks8thwith 2.9 million hectare area under cotton cultivation, the only bio-tech commercial crop of country (James, 2015). Transgenic plants with over expression of *AVP1* gene has resulted in salt and water stress tolerance in *Arabidopsis* (Gaxiola *et al.,* 2001). Plant growth promoting rhizobacteria (PGPR) are important component of microbial population in the rhizosphere of plants including transgenic plants. Among PGPR, bacteria of the genus *Arthrobacter* are ubiquitous in soil and have considerable ability to survive during severe drought conditions (Acosta-Martínez *et al.,* 2014).A stress tolerant phosphate solubilizer *Arthrobacter* strain has been isolated from tomato rhizosphere (Upadhyay *et al.,* 2012). Nitrogen fixing, phytohormone producing*Azospirillum* strains have been extensively studied as inoculants for growth promotion of cereal crops and grasses. It has been reported that*Brevibacillus*strains also mediate plant growth promotion in different crops (Nápoles *et al.,* 2014). We have analyzed the culturable bacterial populations in the rhizosphere of *AVP1*transgenic cotton and compared to its non-transgenic isogenic line. Furthermore, bacteria have been identified *in vitro* that have the potential to promote plant growth and tested *in vivo* as single-strain

inoculum for growth promotion of both transgenic and non-transgenic plants. This study will help to identify the possible risks posed by *AVP1*transgenic cotton on bacterial populations, commercial release of this variety as well as to identify the potential candidate bacteria for development of biofertilizer both for transgenic and non-transgenic cotton in the country.

MATERIALS AND METHODS

Density of bacterial population and isolation of bacteria from *AVP1*transgenic and non-transgenic cotton: *AVP1* transgenic (var. Coker) and non-transgenic cotton (var. Coker) plants were obtained from Gene Transformation Lab, NIBGE Faisalabad, Pakistan (Arshad *et al*., 2013).To investigate bacterial populations, soil samples were collected from rhizosphere of transgenic and non-transgenic cotton plants at different growth stages *i.e*., 30, 60 and 90 days after sowing (DAS). Plants were uprooted carefully and soil adhering to the roots was collected.1.0 grhizospheric soil was added to 9 ml saline solution (0.89%) and 10X serial dilutions up to10^{-5} were prepared. 100 µl soil suspension from each dilution was spread on nutrient agar plates and incubated at 28±2°C for 2-5 days. After incubation, colony forming units (cfu/g) were counted on nutrient agar plates. The representative bacterial colonies were transferred to fresh nutrient agar plates and pure cultures were obtained by repeated streaking on fresh nutrient agar plates and colony morphology i.e. color, shape, size and margins were analyzed. A purified single bacterial colony from each culture was grown in nutrient broth for 72h at 30°C and bacterial cell morphology and motility were examined under microscope at 100 X (Microscope, LaboPhot 2 Nikon, Japan)

Identification of bacteria by 16S rRNA gene sequence analysis: Single bacterial colonies were grown in LB broth at 28±2°C for 72h on an arbitrary shaker. Cells were pelleted from 3ml of bacterial culture by centrifugation at 10,000 × g for 15 min and then cell pellets were washed with TE buffer (10 mMTris.Cl; 1 mM EDTA, pH8).The cell pellets were suspended in 250 µl of TE buffer and placed in boiling water for 5 min. This cell lysate was used as template in each PCR reaction for amplification of 16S rRNA geneas reported by Qaisrani *et al*.(2014).The 16S rRNA gene sequences were processed using Bio-Edit version 7.1 and BLAST searched at NCBI.16S rRNA sequences after identification were deposited to Gen Bank EMBL.

Solubilization of tri-calcium phosphate: Bacterial strains were grown in Pikovskaya broth (50 ml, pH 7) adding 5 gl^{-1} tri-calcium phosphate as insoluble P source (Pikovskaya, 1948). These cultures were grown for 15 days at 28±2°C on a shaker. Supernatant was obtained from bacterial cultures by centrifugation at 4000 × g for 15 min and cell-free supernatant was filtered (0.45 µm filter, Orange Scientific Gyro Disc, Belgium) and processed for quantitative estimation of phosphorus solubilization by molybdate blue color method.

IAA production (indole-3-acetic acid) by bacterial isolates: For quantification of indol-3-acetic acid (IAA), bacterial strains were grown in LB broth. Cell-free culture medium was obtained by centrifugation at 4000 × g, for 15 min, pH was adjusted to 2.8 with HCL (1N) and further sample preparation and analysis on HPLC was followed according to Tahir *et al.*(2013).

Inoculation of transgenic and non-transgenic cotton: Seeds of cotton(*AVP1*and non-transgenic cotton, Coker) were surface-sterilized (0.1% NaOCl) and washed in sterilized distilled water. Cotton seeds were grown in earthen pots, filled with non-sterilized soil (sandy loam soil, pH: 8.4, total N:0.007%, available N:0.0044%, EC: 3.15 m/S, available P:1.85ppm, organic matter 0.006%). Three plants were maintained in each pot with three replicates.

The plants were grown in green house under controlled conditions (28°C,65% relative humidity, photoperiod of 16/8 h light/dark, light flux 1600 lux). Bacterial inoculum for cotton plants was prepared by growing bacterial cultures in nutrient broth (50 ml, at 30ºC for 48h and centrifuged at 4000 × g for 10 min. Bacterial culture (1 ml, ≈10^9cfu/ml) was applied directly near to the root system of cotton seedlings 3 days after germination. 1.8 gof urea and DAP i.e equivalent to recommended fertilizer dose (250 kg/ha of DAP and 250 kg/ha urea) was applied to each pot and data of different agronomic parameters was recorded after harvesting plants at maturity. For measurement of root area, the roots were separated, washed and spread on polyethylene sheet. A computer image of the roots was created after scanning the roots and root length was calculated using root image analysis program (Washington State University Research Foundation program, USA).

Survival of bacterial strains in the carrier materials: Survival of bacterial strains in the carrier material *i.e.*, powdered filter mud (Nitrogen 0.23 %, Phosphorus 0.21%, Potassium 1.26 %, Moisture 45-55%, Humic acids 19.756%, and Sugar 5-12%) was studied for 2 months. The carrier material was autoclaved twice at 121° C and 100 kPa for 20 minutes and packed in falcon tubes (30 g/tube) under sterilized conditions. Bacterial cultures were grown in nutrient broth medium at 30°C on shaker for 48 hours. In carrier material, 10 mlof bacterial culture were added. Three replicates of each culture were prepared. Inoculated tubes were placed at 30±2°C. Microbial population was estimated in each treatment at different intervals up to 60 days using serial dilution plating technique on nutrient agar plates and colony forming units (log cfu/g carrier material) were calculated

Statistical analysis: Experiment was laid out in Completely Randomized Design (CRD) with three replicates. Results were subjected to analysis of variance (ANOVA) and significance at 5% level was tested by LSD test using a computer software program. Mean values and the standard error were calculated**.**

RESULTS AND DISCUSSION

Increase in the adoptability and cultivation of genetically modified crops demands well-defined risk assessments. Transgenic plants are known to have effect on the abundance, diversity and activity of soil microorganisms living in close proximity with their roots (Mina *et al.,* 2007). The effects of transgenic plants on the soil and ecosystem function should be carefully evaluated before the release of any transgenic plant variety. In the present study, we investigated the bacterial populations in the rhizospheric soil of *AVP1* transgenic and non-transgenic cotton plants on nutrient agar medium (cfu/g soil) at three crop developmental stages (30, 60, and 90 DAS). No significant difference was observed among bacterial populations, at all three plant developmental stages of *AVP1* transgenic and non-transgenic cotton plants. However fluctuation in the rhizospheric bacterial population (cfu) was recorded that showed relatively low populations at 30 DAS compared with 60 and 90 DAS in the rhizosphere of transgenic as well as non-transgenic cotton plants (Fig. 1). This indicated that *AVP1* gene did not alter cultureable bacterial populations in the rhizosphere. Studies made on transgenic potato (T4 lysozyme-expressing transgenic potato) have shown that general environmental parameters (cultivar and soil type, seasonal and climate changes, plant age) have more marked effect on the rhizosphere microflora than the presence of the transgenic character (Heuer *et al.,* 2002; Lottmann *et al.,* 1999, 2000). In the present study, regardless of transgenic character, a significant variation in bacterial population was observed with the crop development stage. This may be the effect of different substrates released by plants roots as exudates which varied qualitatively and quantitatively during different plant developmental stages (Nannipieri *et al.,* 2008). Previous studies indicated that changes in rhizosphere population and microbial diversity may occur as a plant matures (Kertesz and Mirleau, 2004).

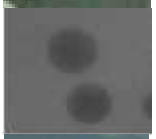
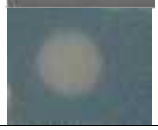
During this study three bacterial strains Bα (*Arthrobacter*sp.), BM31 (*Azospirillum*sp.) and TN4-3NF (*Brevibacillus*sp.) with distinct cell shape, motility and colony morphology were isolated from *AVP1* transgenic and non-transgenic cotton rhizosphere soil. 16S rRNA gene sequence analysis of bacterial isolates revealed that these isolates belonged to bacterial genera *Arthrobacter*, *Azospirillum* and *Brevibacillus* (Table 1).Isolation of *Arthrobacter* strains from the rhizosphere of various crops has been reported. Bacterial strains belonging to genus *Bacillus* and related genus *Brevibacillus* colonize the cotton root zone and use different mechanisms to affect the plant growth(Ahmad and Kibert, 2014). Isolation of *Azospirillum* has frequently been reported from rhizosphere of grasses like cereals and cotton as well (Yasmin *et al.,* 2013).Phosphate solubilization by bacteria is an important mechanism utilized for plant growth promotion. The bacterial isolates were tested for their phosphate solubilization activity in pure culture grown in Pikovskaya medium. Maximum phosphate solubilization activity was recorded in pure culture of *Brevibacillus* strain TN4-3NF (46.02 µg/ml), followed by *Arthrobacter* strain Bα (36.74 µg/ml) and *Azospirillum* strain BM31(12.34 µg/ml) (Table 2). P-solubilization ability of different strains of *Brevibacillus* has been reported recently (Nápoles *et al.,* 2014) and also other bacterial genera including *Arthrobacter*, *Azospirillum* and *Bacillus* (Bhattacharyya and Jha, 2012; Tahir *et al*., 2013; Qaisrani *et al.,* 2014). It has been estimated that more than 80% of bacteria isolated from the rhizosphere can produce plant growth regulator IAA (Khalid *et al.,* 2005). Indol-3-acetic acid (IAA) production ability of isolates was investigated in pure culture grown in tryptophan-supplemented LB broth and tryptophan-free medium. Maximum IAA production (121.75 µg/ml) was shown by *Azospirillum* strain BM31, followed by *Brevibacillus* strain TN4-3NF (72.15 µg/ml) and *Arthrobacter* strain Bα (39.06 µg/ml) in tryptophan-supplemented medium. Considerably less amounts of IAA were detected in the tryptophan-free growth medium of all strains tested in the present study (Table 2). This trend of low amount of IAA production by PGPR in tryptophan-free growth medium has been reported (Hassan *et al.,* 2014). Many strains of *Azospirillum* produce IAA in considerable amount that favors the plant growth. *Arthrobacter* strains have been reported for IAA production under stress conditions (Banerjee *et al.,* 2010).

Use of PGPR with the aim of improving nutrient availability for plants and for sustainable agriculture has been studied during past couple of decades (Habig *et al.,* 2015). A pot experiment was conducted to study the effect of PGPR strains isolated from cotton rhizosphere. All the inoculated treatments showed positive effect on growth parameters of transgenic and non-transgenic cotton plants when compared with non-inoculated treatments. *Arthrobacter* strain Bα, *Azospirillum* strain BM31 and *Brevibacillus* strain TN4-3NF were used as single-strain inoculant for both transgenic and non-transgenic cotton plants. Among the tested strains, inoculation of *Brevibacillus* strain TN4-3NF showed maximum impact on cumulative root length, shoot dry weight, root dry weight and yield of both transgenic and non-transgenic cotton plants. Inoculation of this strain to non-transgenic cotton plants showed maximum improvement of cumulative root length (14.7%), shoot

dry weight (16.5%), root dry weight (17.6%), and yield (13.9%) over non-inoculated control. Inoculation of transgenic cotton with *Brevibacillus* strain TN4-3NF resulted in increased cumulative root length (11.9%), shoot dry weight (5.54%), root dry weight (14.2%), and yield (13.4%) over non-inoculated control (Table 3). The strains of *Brevibacillus* positively affected egg-plant and pepper plantlets germination and development under organic treatment, corroborating its plant growth promoting traits (Nápoles *et al.,* 2014). Different strains belonging to the genera tested in this study have been reported as PGPR inoculant of cotton plants as well as other crops (Mirza *et al.,* 2006; Kamal *et al.,* 2014). It has been reported that plant growth promotion of transgenic cotton (*Bt*) was regulated by inoculation of different strains of *Arthrobacter* and *Brevibacillus* (Barazani and Friedman, 2000).Survival of the bacterial isolates was also tested in powdered filter mud which is used as carrier material for commercial scale production of bacteria-based biofertilizer. All three strains showed very good survival ability in the carrier material tested up to two months after inoculation (Fig. 2). After two months, the number of colony forming units (log cfu/g of dry weight)obtained for *Arthrobacter* strain Bα were 6.37, *Azospirillum* strain BM31 were 6.5 and *Brevibacillus* strain TN4-3NF were 6.55 in the carrier material. Excellent survival of *Brevibacillus* may be due to its spore forming ability under stress conditions (Kumar *et al.,* 2011).

Biosafety is a major concern prior to introduction of any transgenic plants in the agriculture system. In the present study culturable bacterial populations and interaction of plant growth promoting rhizobacteria with transgenic cotton plants were studied to record any effect of transgenic plants on rhizospheric bacteria. We observed that culturable bacterial populations of *AVP1* transgenic and non-transgenic cotton were similar and effect of bacterial inoculations on plant growth was comparable. However detailed biosafety studies on other microbes and animal model systems may be conducted before release of transgenic plants to farmers

Table 1. Colony and cell morphologies and identification of the bacterial isolatesfrom the rhizosphere of *AVP1* transgenic and non-transgenic cotton

Isolate	Colony and cell morphology	Colony on Nutrient agar	Accession No.	Maximum *16SrRNA* similarity in NCBI
1. B-α	White colonies, cells slightly motile, Gram variable rods		HE995801	99 % *Arthrobacter oxydanse* (KC934793.1)
2. BM31	Off-white colonies, Gram negative cells with spiral motility		HE995805	99% *Azospirillum brasilense* (KC920689.1)
3. TN4-3NF	Milky white colonies, cells motile Gram positive rods		HE995803	100% *Brevibacillus laterosporus* (KF973294.1)

To study the colony and cell and colony morphology, pure bacterial cultures were grown in LB broth medium for 48 h at 30° C. Cell supernatant (20µl) of bacterial culture were examined under light microscope. For identification of bacteria, 16S rRNA gene was amplified through PCR and PCR product was sequenced.

Table 2. Quantification of IAA production and P-solublization ability of bacterial strains isolated from cotton

No.	Bacterial Strains	*IAA (µg/ml) supplemented with tryptophan	**IAA (µg/ml) without tryptophan	***Available P (µg/ml)
1	*Arthrobacter* sp. strain Bα	39.06±11.5	7.2±0.5	36.74±8.5
2	*Azospirillum* sp. strain BM31	121.75±12.8	10.3±1.1	12.34±1.2
3	*Brevibacillus* sp. strain TN4-3NF	72.15±8.2	6.3±1.1	46.02±5.2

*Indol-3-acetic acid (IAA) produced in the cell free culture medium supplemented with tryptophan. .

**Indol-3-acetic acid (IAA) produced in the cell free culture medium supplemented without tryptophan.

***Phosphate solubilization (µg/ml) by bacterial isolates in Pikovskaya media containing insoluble tri-calcium phosphate. The values given in the table are an average of three determinations with standard deviation.

Table 3. Effect of bacterial inoculation on growth of *AVP1*-transgenic and non-transgenic cotton grown in earthen pots under controlled conditions

Treatment	Cumulative Root Length (cm)		Root Dry weight (g)		Shoot dry weight (g)		Yield (Lint + seed)	
	Transgenic	Non-transgenic	Transgenic	Non-transgenic	Transgenic	Non-transgenic	Transgenic	Non-transgenic
Control	53.50^{BC}	48.86^{DE}	8.31^{CD}	8.27^{D}	21.93^{CD}	20.32^{D}	10.66^{B}	10.49^{C}
Arthrobacter strain Bα	56.14^{AB}	46.0^{E}	8.50^{BCD}	8.69^{BC}	22.26^{BC}	22.73^{BCD}	11.68^{AB}	11.81^{AB}
Azospirillum strain BM31	56.33^{AB}	52.33^{BC}	9.78^{A}	8.52^{BCD}	24.59^{AB}	24.75^{AB}	12.28^{A}	10.57^{BC}
Brevibacillus strain N4-3NF	58.90^{A}	56.10^{AB}	9.80^{A}	8.69^{BC}	25.98^{A}	24.34^{AB}	12.54^{A}	11.95^{A}
LSD(P≤ 0.05)	4.37		1.02		2.7		1.75	

Values in same column sharing the same letter do not differ significantly (P≤ 0.05) according to Fisher's LSD, (n=3). Plants were maintained till maturity and one ml bacterial cultures (≈10^9cfu/ml) were inoculated to seedlings after emergence. Three seedlings were maintained in each pot with three replicates.

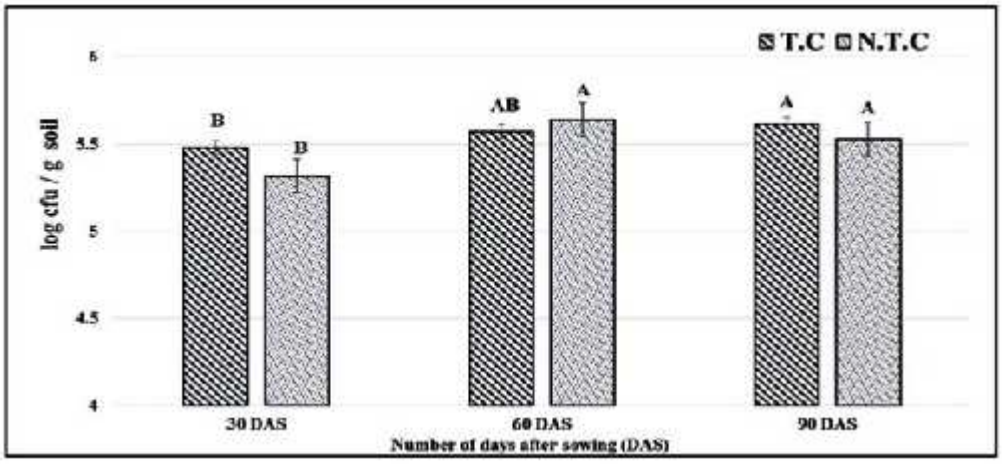


Figure 1. Bacterial population (log cfu/g soil) in the rhizosphere of *AVP1*-transgenic (TC) and non-transgenic cotton (NTC) at 3 different plant growth stages (30, 60 and 90 days after sowing). Values are the mean of three replicates. Letters A and B on bars represent statistically different values at 5% level

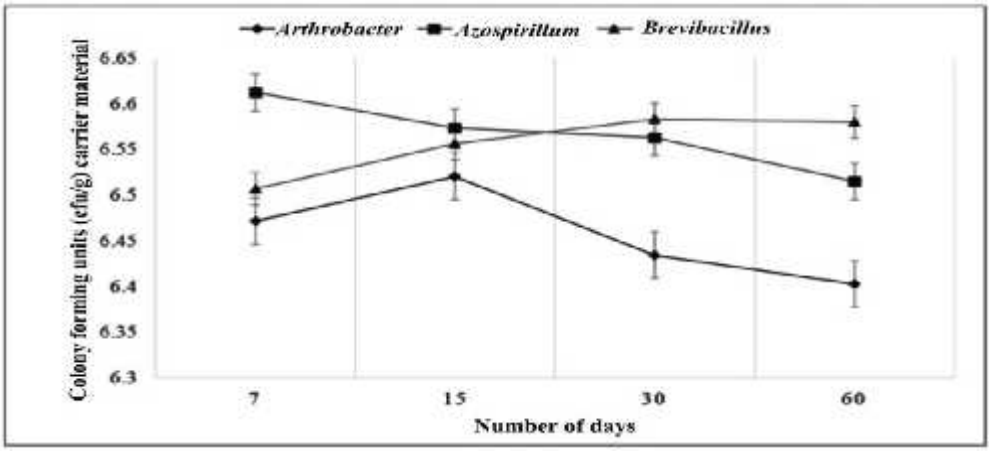


Figure 2. Bacterial population (cfu/g filter mud) at different days i.e., 7, 15, 30 and 60 days after inoculation of carrier material (filter mud). The values are average of three replicates. Error bars show the standard deviation of mean within the sample

Conclusions: This study showed that populations of culturable bacteria in the rhizosphere of transgenic and non-transgenic cotton as well as response to bacterial inocula are similar. It points to safe use of transgenic plants for larger scale cultivation.

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