Pak. J. Agri. Sci., Vol. 51(1), 91-99; 2014
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

ISOLATION AND IDENTIFICATION BY 16S *RRNA* SEQUENCE ANALYSIS OF *ACHROMOBACTER, AZOSPIRILLUM* AND *RHODOCOCCUS* STRAINS FROM THE RHIZOSPHERE OF MAIZE AND SCREENING FOR THE BENEFICIAL EFFECT ON PLANT GROWTH

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The aim of the present study was to isolate and characterize plant growth promoting rhizobacteria from the rhizosphere of field-grown maize. Among the five bacterial isolates obtained in the present study, the isolates M1, M7 and ZN1 showed typical *Azospirillum*-like cell morphology, motility and high sequence similarity of 16S rDNA to the genus *Azospirillum*. The isolates M25 and M28 were identified on the bases of 16S rRNA sequence analysis as *Achromobacter* and *Rhodococcus*, respectively. All the five isolates obtained in the present study produced phytohormone IAA in pure culture but phosphate-solubilization activity was detected only in pure cultures of *Achromobacter* strain M25 and *Rhodococcus* strain M28. The isolates were tested as inoculants for maize seedlings grown in sterilized sand from which cotyledons were removed at an early growth stage to deprive the seedlings of internal nutrient source and harvested after 5 weeks. For comparison, seedlings with intact cotyledons were used as control. In addition inoculated plants were grown in non-sterilized soil in earthen pots and harvested at maturity. In both the short-term inoculation experiments carried out in sterilized sand as well as in long-term inoculation experiment in earthen pots all the inoculated strains improved plant growth compared to respective non-inoculated controls. However, unlike the seedlings with intact cotyledons, the pattern regarding the efficiency of growth promotion by inoculated strains (*Azospirillum* strains ZN1>M7>M1>*Achromobacter* strain M25>*Rhodococcus* strain M28) was the same in plants with detached cotyledons and the plants grown to maturity in non-sterilized soil. It can be concluded from the short-term experiments under controlled conditions that removal of cotyledons helps effective screening of bacterial inocula compared to seedlings with intact cotyledons.

Keywords: inoculation studies, Rhizospheric bacteria, plant beneficial traits, phytohormone IAA, phosphate solubilization, cotyledons,

INTRODUCTION

Being the highest yielding cereal crop, maize is emerging as an alternative food source for the third world countries after wheat and rice. Due to availability of high yielding hybrid varieties, maize has become a valuable resource for wet-milling industry and livestock feed. Maize makes a greater quantity of epigeous mass than other cereal plants. For the same reason it is extensively used as fodder and as a feedstock for biogas plants in some advanced countries (Pimentel and Patzek, 2005). The major constituents of maize seeds include carbohydrates, sugars, dietary fiber, fats, proteins, amino acids, vitamins and minerals like iron, magnesium and potassium. These seed reserves are utilized by seedling during germination and early growth stages, followed by an establishment period that ends when the seedlings have exhausted the food reserves stored in the seed. At this stage the growth rate is determined by root nutrient uptake from surrounding environment (Deleens *et al.*, 1984; Whalley *et al.*, 1966). The maize endosperm is a major storage house of seed nitrogen (Harvey and Oaks, 1974) as storage proteins, zein and glutelin. The other major plant nutrient phosphorus is stored primarily in the form of phytic acid in seeds (Greenwood and Batten, 1995; Park *et al.*, 2006).

Except for initial heterotrophic growth period during which it solely draws on seed resources, the plants remain dependent upon soil nutrients throughout the growth period. For most agricultural crops, additional nutrients like nitrogen (N), phosphorous (P) and potassium (K) are applied as chemical fertilizers to get maximum yields. Rhizospheric bacteria have been implicated in mineral nutrition of plants by facilitating their availability (Figueiredo *et al.*, 2011). These plant growth promoting rhizobacteria (PGPR) utilize multiple mechanisms like biological nitrogen fixation and phosphate solubilization and contribute to N and P nutrition of the plant. Nitrogen fixing bacteria (the diazotrophs) reduce atmospheric nitrogen (N_2) into plant useable form ammonium. P-solubilizing microbes convert unavailable form of orthophosphate into soluble forms, suitable for plant

uptake (Hinsinger, 2001). In addition PGPR express plant beneficial traits like production of phytohormones and bio-control agents (Lugtenberg and Kamilova, 2009). A single PGPR may exhibit one or more than one growth promoting mechanisms. PGPR are a heterogeneous group and include bacterial genera like *Azospirillum, Herbespirillum, Pseudomonas, Bacillus, Azotobacter* and *Bulkhorderia* (Gray and Smith, 2005). Applications of single-strain inocula or bacterial consortia have been reported to promote growth in many agricultural crops including maize (El-Katatny, 2010; Marques *et al.*, 2010; Nadeem *et al.*, 2006). However, results of screening of bacterial inocula under controlled conditions often contradict the observations made under natural conditions. Ramazan *et al.* (2006) reported up to 46.7% increase in root weight under greenhouse conditions but under field conditions the increase was 16.7% in sugar beet. Similar results of bacterial inoculation have been reported in maize (Morales-Garcia *et al.*, 2011). The increase in dry weight of maize plantlets was 110% over non inoculated control under controlled conditions while in plants under natural condition 52% increase in kernel biomass was observed.

The present study deals with the isolation of bacterial strains from the rhizosphere of maize, followed by their identification based on 16S rRNA sequence analysis and detection of indole acetic acid (IAA) and phosphate solubilization in pure cultures. The effect of bacterial inocula on plant growth was investigated in short-term experiments by removing cotyledons at an early growth stage to deprive the seedlings of internal nutrient source and thus attempt was made to restrict seedlings on external nutrient sources including contribution from inoculated PGPR. In addition, the plants were inoculated and grown to maturity under natural conditions in earthen pots.

MATERIALS AND METHODS

***Isolation of bacterial strains*:** Roots and rhizosphere soil samples were collected from the maize variety FSH-421 growing in a farmer's field at Faisalabad (73°74 East, latitude 30°31.5 North, with an elevation of 184 meters above sea level). For isolation of bacteria nitrogen free malate (NFM) semi-solid medium (Okon *et al.*, 1977) was used. Root pieces (5-10 mm length) along with rhizospheric soil were added to 1 mL semi-solid NFM medium in eppendorf tubes. After two days incubation at 28°C, 50 µL from each tube were transferred to a fresh eppendorf tube containing semi-solid NFM. This procedure was repeated five to six times to get enriched cultures and then a loopful was streaked on Luria Bertani (LB) and NFM agar plates. Single colonies were picked from these plates and streaked on fresh LB plates. The bacteria were further purified by repeated sub-culturing on LB agar plates. Colony morphology of purified bacterial isolates was studied by streaking on NFM and LB agar plates, grown at 28 ± 2ºC for 24 h. The shape and motility of bacterial strains were observed under light microscope (Model, Nikon LABOPHOTO-2, Japan).

***IAA production by bacterial isolates*:** Quantitative estimation of IAA produced by bacterial cultures was carried out on HPLC (Tien *et al.*, 1979). Bacterial isolates were grown in a conical flask containing 50 mL LB broth supplemented with L-tryptophan (100 mg L^{-1}). Cell free supernatant was obtained by centrifuging at 6000 rpm for 10 min and pH of the supernatant was adjusted to 2.8 with 1N hydro chloric acid (HCl). Auxins from supernatant were extracted by using equal volume of ethyl acetate. Extract was evaporated till dryness and re-suspended in 1 mL of methanol. The samples were analyzed by HPLC (Varian Prostar 210, USA) using UV detector and C-18 column. Methanol: acetic acid: water (30:1:70) was used as mobile phase at the rate of 0.6 mL min^{-1} (Rasul *et al.*, 1998). Pure IAA was used as standard. IAA identification and quantification was done by comparing the retention time and peak area by computer software (Varian).

***Phosphate solubilization by bacterial isolates*:** Phosphate solubilizing ability of the bacterial strains was studied on Pikovskaya's agar (Sigma, USA) containing tricalcium phosphate (TCP) as insoluble P source (Pikovskaya, 1948). Pikovskaya agar plates were incubated for one week at 28 ± 2ºC and observed for the formation of transparent halo zone around the colonies of inoculated bacteria. Phosphate solubilization by bacterial isolates was quantified by spectrophotometric method (Watanabe and Olsen, 1965). Bacterial cultures were grown in 100 mL Pikovskaya broth medium for 2 weeks at 30ºC and constant shaking @ 120 rpm (Kuhner shaker, Switzerland). Flasks, containing the same media but without inoculation, were treated as blank. pH of the medium was recorded with a pH meter equipped with a glass electrode. Cell-free supernatant was obtained by centrifuging at 6000 rpm and used for measuring soluble phosphorous by Mo-blue method on spectrophotometer (Camspec M350 double beam UV visible, UK.).

***Extraction of total genomic DNA*:** Total genomic DNA from pure bacterial strains was extracted by the CTAB method (Wilson, 1987) with some modifications. Bacterial cultures were grown in LB broth for 24 hours at 30 ± 2°C with constant shaking (150 rpm). The cells from pure cultures were harvested by centrifugation at 13000 rpm for 2 min and pellet was re-suspended in 567 µL of TE buffer in eppendorf tube. After addition of 30 µL sodium dodecyl sulfate (SDS) (10% w/v) and 3 µL proteinase K (20 mg mL^{-1}), the contents of eppendorf were mixed thoroughly and incubated for one hour at 37°C. To this suspension, 100 µL 5 M NaCl and 80 µL CTAB [Cetyltrimethyl ammonium bromide; 10% CTAB/0.7 M NaCl] was added, mixed and incubated for 15 minutes at 65°C. The mixture was centrifuged at 13000 rpm for 5 min and supernatant was

extracted twice with chloroform/isoamyl alcohol (24:1) followed by extractions with phenol/chloroform/isomyl alcohol (25:24:1). To the aqueous layer, double volume of absolute ethanol was added and incubated at -20°C for 1 h. After centrifugation at 13000 rpm for 10 min, pellet was washed with 70% ethanol before drying under vacuum and dissolved in 30 µL of double distilled de-ionized water.

***PCR amplification*:** To confirm that the three *Azospirillum* isolates are different and not the re-isolate of the same strain, a random primer OPI6 (AAGGCGGCAG) was used in PCR and the banding patterns of PCR products were compared on 1% agaros gel. PCR conditions used were same as given below for amplification of 16S rRNA gene, except that the annealing temperature was 38°C and primer concentration was 2.5 µM.

For the amplification of 16S rRNA gene conserved primers PH (AAGGAGGTGATCCAGCCGCA) and PA (AGAGTTTGATCCTGGCTCAG) (Edwards *et al.*, 1989) were used. A reaction mixture of 25 µL containing 20 ng template DNA, 2.5 µL 10X *Taq*polymerase buffer (Fermentas), 0.5 µL 10 mM dNTPs (Fermentas), 2 µL of 25 mM $MgCl_2$, 0.5 µM each of primers and 0.2 units *Taq*DNA polymerase (Fermentas) was prepared in a 0.5 mL thin-walled PCR tube and used for PCR. The reaction mixture was incubated in a thermal cycler (Eppendorf, Germany) and the conditions for PCR amplification were 94°C for 5 min for initial denaturation, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

***Phylogenetic analysis*:** Sequences of 16S *rRNA* of all isolates were blast searched using NCBI data base. Alignment was performed on CLUSTAL X (Thompson *et al.*, 1997) by using the strains isolated in this study and their closely related sequences downloaded from the NCBI databank. Maximum likelihood (ML) method was adopted to analyze these sequences as described by Mirza *et al*. (2009). Bootstrap value of 70 or greater was kept as representative (Hillis and Bull, 1993).

***Preparation of bacterial inoculum*:** The bacterial strains were grown in 50 mL of LB for 24 h at 28 ± 2°C. Cells were harvested by centrifugation at 5000 rpm and re-suspended in equal volume of 0.85% saline for inoculation. Maize variety FSH-810 was used to screen bacterial isolates for plant growth promotion. For growing plants, sand was soaked in 0.5 N nitric acid (HNO_3) for one day and washed thoroughly to remove acid. Sand was then dried and autoclaved. Maize seeds were sterilized in sodium hypochlorite (3%) for 3 min followed by a brief washing with 70% ethanol and finally thoroughly washed with sterilized water. A single maize seed was sown in 50 mL Falcon tube filled with sterilized sand containing TCP@ 5 g Kg^{-1} and placed in the growth room. The plant growth conditions were set at 16⁄8 h light/dark cycle and 28⁄20°C day⁄night temperature. The seedlings were irrigated with diluted Hoagland solution ($1/10^{th}$ strength). Plants were gently removed from the Falcon tubes 4 days after germination, cotyledons were removed using a sharp sterilized blade and re-planted. Seedlings with intact cotyledons as well as the seedlings from which cotyledons were removed, received 1 mL (approximately 10^9 cells) of inocula. Plants were harvested after five weeks of germination and plant dry weight was recorded.

For pot experiment, the soil was ground and sieved through 2 mm sieve. Earthen pots were filled with 20 Kg soil (sandy loam, EC 2.5 dsm^{-1}, pH 8.2, organic matter 0.6%, available phosphorous 7.5 mg Kg^{-1} and total nitrogen 0.06%). Seeds of maize (250 g), powdered and dried filter mud (25 g) and inocula (50 mL) were mixed by gentle shaking until the seeds were covered with a thin layer of inoculum and filter mud. Three inoculated seeds were sown in each pot. After germination single plant was maintained and the remaining seedlings were removed. Recommended dose of nitrogen (140 Kg ha^{-1}) phosphorous (100 Kg ha^{-1}) and potassium (70 Kg ha^{-1}) was applied to each pot. Plant dry weight and grain weight was recorded after 90 days of sowing at maturity.

***Statistical analysis*:** Effect of different bacterial isolates on different growth parameter of maize was determined through analysis of variance (ANOVA) using STATISTIX 8.1 software. Means were compared by applying least significant difference test (LSD) at alpha 0.05 on all the parameters.

RESULTS

***Isolation and morphological characterization of bacterial isolates*:** Five bacterial isolates were obtained from the rhizosphere of maize (Table 1). The isolates ZN1 and M7 formed off-white colonies on LB agar plates and the cells were vibroid motile rods resembling *Azospirillum* in semi-solid NFM medium (Krieg and Dobereiner, 1984). The isolate M1 formed pinkish colonies on LB agar plates and the cell morphology and motility was similar to those of isolates ZN1 and M7. The isolate M25 formed milky white round colonies and cells were highly motile short rods. The colonies of M28 were orange pigmented and the cells were long rods.

***Bacterial differentiation by RAPD analysis*:** The three isolates (M1, M7 and ZN1) showing morphological resemblance to *Azospirillum* were differentiated (Fig. 1) by comparing banding patterns of PCR products obtained by using a random primer. All three strains showed different banding pattern of the PCR product on agarose gel. Seven bands were observed in the isolate ZN1 with three strong bands of 800, 1400 and 2800 bp. In the isolate M7 five DNA bands including two very strong bands at position 1100 and 1300 bp were detected. Six DNA bands were obtained from the isolate M1 with one very strong band of approximate size 1700 bp.

Table 1. Morphology of the bacterial isolates from maize and their identification on the basis of 16S rRNA sequence analysis

Isolates	Similarity	Similarity (%)	Colony Morphology	Cell Morphology
ZN1	*Azospirillum brasilense* strain MTCC4035 Acc. No. Q288686.1	98	Off white, Round	Vibroid motile rods
M1	*Azospirillum brasilense* strain Gr62 Acc. No. FR745918.1	98	Pink, Round	Vibroid motile rods
M7	*Azospirillum brasilense* strain Gr62 Acc. No FR745918.1	99	Off white, Round	Vibroid motile rods
M25	*Achromobacter* sp. NCW Acc. No EU220009	98	Milky white, Round	Short motile rods
M28	*Rhodococcus* sp. Z25 Acc. No FJ752527.1	98	Orange, Round	Long motile rods

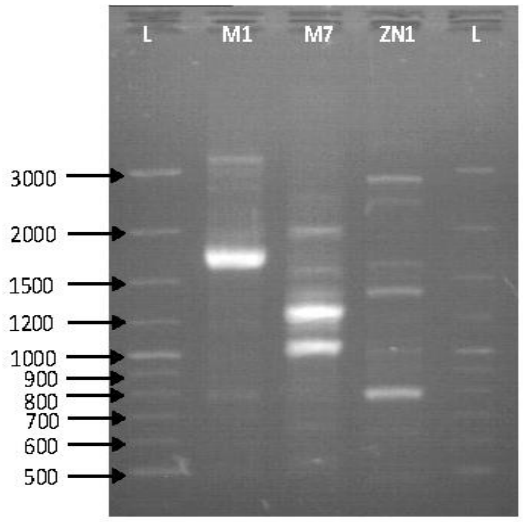

Figure 1. Differentiation of *Azospirillum* strains (M1, M7 and ZN1) by comparison of the banding pattern of RAPD PCR products. L= DNA marker.

***Identification of bacterial isolates by 16S rRNA and Phylogenetic analysis*:** The identification of these isolates from maize was further confirmed by 16S rRNA sequence analysis (Table 1). After PCR amplification of 16S rRNA genes, DNA sequences were obtained and compared with GenBank sequence utility (www.ncbi.nlm.nih.gov). The data showed 98% sequence similarity of strain ZN1 with *Azospirillum brasilense* strain MTCC4035. The sequences of both the isolates M1 and M7 showed 98% and 99% sequence homology with *A. brasilense* strain Gr62. Phylogenetic tree (Fig. 2) showed clustering of strains M1, M7 and ZN1 with *Azospirillum brasilense*. The isolate M25 showed 98% sequence similarity with *Achromobacter* sp. NCW and M28 showed high similarity with *Rhodococcus* sp. Z25. In phylogenetic tree (Fig. 3 and 4) isolates M25 and M28 showed close relatedness to *Achromobacter xylosoxidans* and *Rhodococcus rhodochrous*, respectively.

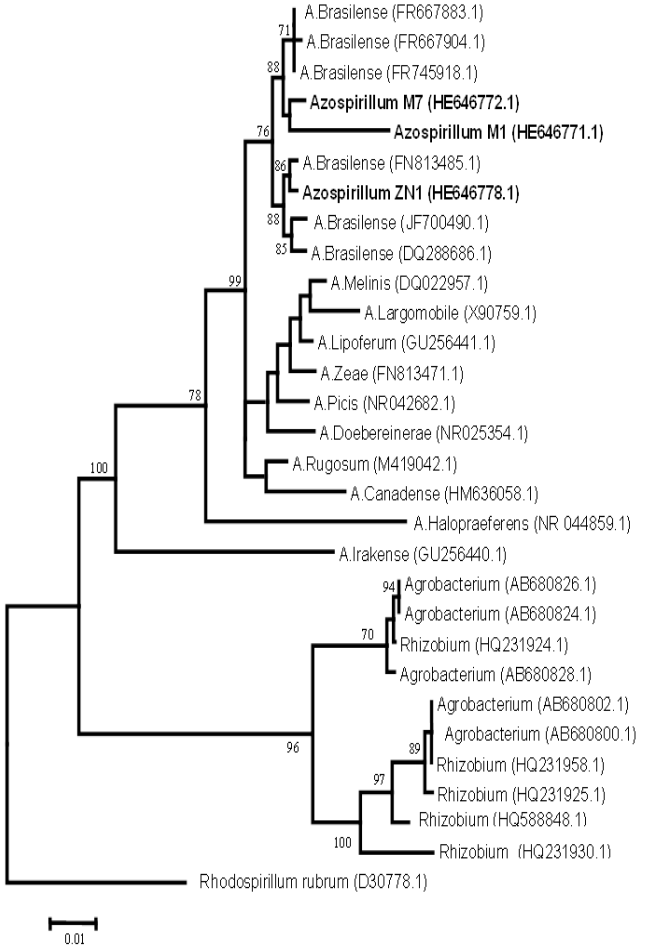

Figure 2. Maximum likelihood-based tree showing the phylogenetic relationship of the *Azospirillum* strains based on the sequences of the 16S rRNA gene. *Azospirillum* strains isolated in the current study (presented in bold) along with those of the closely related sequences obtained from NCBI databank. Numbers above the nodes represent maximum likelihood bootstrap support above 70%.

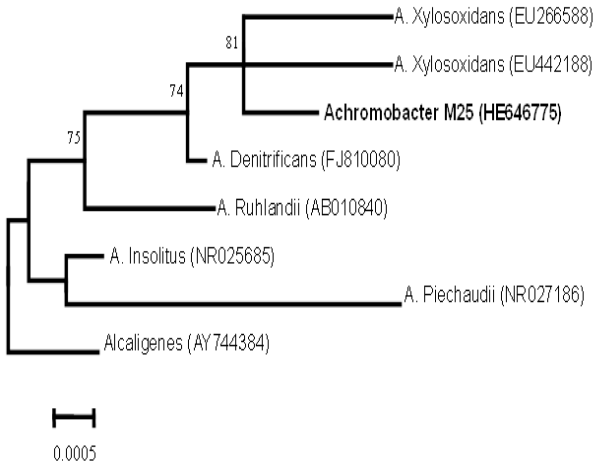

Figure 3. Maximum likelihood-based tree showing the phylogenetic relationship of the *Achromobacter* strains based on the sequences of the 16S rRNA gene. *Achromobacter* strains isolated in the current study (presented in bold) along with those of the closely related sequences obtained from NCBI databank. Numbers above the nodes represent maximum likelihood bootstrap support above 70%.

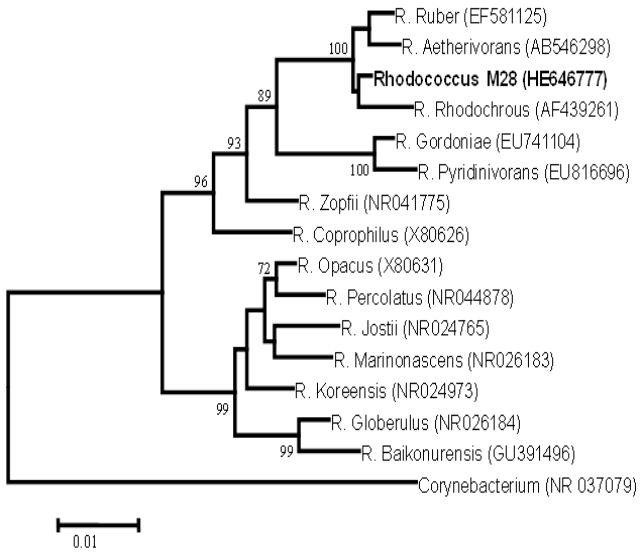

Figure 4. Maximum likelihood-based tree showing the phylogenetic relationship of the *Rhodococcus* strains based on the sequences of the 16S rRNA gene. *Rhodococcus* strains isolated in the current study (presented in bold) along with those of the closely related sequences obtained from the NCBI databank. Numbers above the nodes represent maximum likelihood bootstrap support above 70%.

***Production of IAA and phosphate solubilization by bacterial isolates*:** The three *Azospirillum* strains isolated from maize produced higher amount of IAA compared with *Achromobacter* and *Rhodococcus* strains (Table 2) *Azospirillum* strain ZN1 produced maximum amount of IAA (23.9 mg L^{-1}) while *Achromobacter* and *Rhodococcus* produced 11.6 and 11.5 IAA mg L^{-1}, respectively.

Table 2. Production of IAA and phosphate solubilization by the bacterial isolates from maize

Bacterial strains	IAA (mg/L)*	Available P (mg/L)**
Azospirillum brasilense strain ZN1	23.98 ± 0.7	-ve
Azospirillum brasilense strain M1	23.38 ± 0.4	-ve
Azospirillum brasilense strain M7	22.27 ± 0.4	-ve
Achromobacter sp. strain M25	11.6±0.4	105.8 ±0.4
Rhodococcus sp. strain M28	11.51±0.6	59.0 ± 0.6

* Bacterial cultures were grown in LB containing tryptophan as precursor of IAA biosynthesis; **Bacterial cultures were grown in Pikovskaya medium containing TCP as insoluble phosphorous.

Halo zone formation, indicative of P-solubilization ability of bacteria, was not observed around colonies of *Azospirillum* strains on Pikovskaya agar plates. Halo zone formation was observed around colonies of *Achromobacter* strain M25 and *Rhodococcus* strain M28. Phosphate solubilization ability of isolates quantified by using spectrophotometer showed that only *Achromobacter* and *Rhodococcus* were capable of phosphate solubilization in Pikovskaya broth and all the three *Azospirillum* strains lacked this ability (Table 2). Conversion of TCP into soluble phosphate forms was much higher (105.8 mg L^{-1}) in *Achromobacter* strain M25 compared to *Rhodococcus* strain M28 (59.0 mg L^{-1}).

***Screening of bacterial isolates for plant growth promotion*:** For screening of bacterial isolates as inoculants for growth promotion of maize, cotyledons of the seedlings grown under controlled conditions were detached four days after germination and inoculated with bacterial cultures. Plants were harvested at the age of five weeks and it was observed that control (un-inoculated) plants from which cotyledons had been detached produced less dry weight compared to the plants with intact cotyledons (Table 3). However comparison of inoculated treatments indicated that the plants from which cotyledons were detached responded more positively to applied inoculum than plants with intact cotyledons. All the inoculated strains increased plant dry weight in both the cotyledons-detached and with intact-cotyledon plants than their respective non-inoculated controls. In case of cotyledon-detached plants, more plant dry weight was observed in plants inoculated with the

Azospirillum strain ZN1, followed by *Azospirillum* strain M7. Inoculation with *Azospirillum* strain M1 also increased plant dry weight compared to non-inoculated control. Compared with the seedlings inoculated with *Azospirillum* strains, less increase in plant dry weight was observed in plants inoculated with *Achromobacter* strain M25 and *Rhodococcus* strain M28. It was observed that the inoculated plants from which cotyledons were removed showed a statistical difference among treatments (Table 3). Bacterial inoculation of plants with intact cotyledon increased plant growth as reflected by increase in dry weight compared with control (Table 3). However, no significant difference was found among the plants inoculated with different strains.

Table 3. Effect of inoculation with different PGPR strains on maize seedlings with intact and detached cotyledons

Treatments	Dry weight (g) intact cotyledons	Dry weight (g) detached cotyledons
Control	0.58 B	0.52 D
Azospirillum strain ZN1	0.72 A	0.65 A
Azospirillum strain M1	0.72 A	0.61 B
Azospirillum strain M7	0.72 A	0.66 A
Achromobacter strain M25	0.70 A	0.57 C
Rhodococcus strain M28	0.69 A	0.57 C

* There were six replicates for each treatment and LSD was applied at alpha 0.05

All the five bacterial isolates from maize were tested as inoculants for maize plants grown in non-sterile soil in a pot experiment (Table 4). All the strains increased the plant dry weight as compared to control. As reflected by the plant dry weight, maximum growth promotion was observed in plants inoculated with *Azospirillum* strains followed by *Achromobacter* strain. *Rhodococcus* strain M28 had comparatively less impact on plant dry weight of inoculated plants compared to other strains. All isolates increased grain yield as compared to un-inoculated control plants. Maximum grain yield was recorded in case of treatment inoculated with *Azospirillum* strain ZN1 i.e., 70.23 g and minimum grain yield was observed in case of un-inoculated control i.e, 58.01 g.

DISCUSSION

Five bacterial isolates were obtained from the rhizosphere of maize. Among these, three isolates ZN1, M1 and M7 formed vibroid rods and the cell motility in N-free NFM was very much similar to typical azospirilla (Krieg and Dobereiner, 1984). All the three isolates exhibited unique banding patterns of PCR products obtained by using a random primer (Hadrys *et al.,* 1992). This indicated that all three isolates were different and not the re-isolate of the same

Table 4. Effect of bacterial inoculants on growth of maize growing in non-sterlized soil

Treatment	Plant dry weight (g)	Grain yield per Plant (g)
Control	46.8 D	58.01 C
Azospirillum strain ZN1	58.65 AB	70.23 A
Azospirillum strain M1	55.98 AB	70.06 A
Azospirillum strain M7	59.76 A	70.12 A
Achromobacter strain M25	55.31 B	65.8 AB
Rhodococcus strain M28	51.31C	64.48 B

* There were six replicates for each treatment and LSD was applied at alpha 0.05

Azospirillum strain. The identification of the three isolates resembling *Azospirillum* was further confirmed by 16S *rRNA* sequence analysis. All the three isolates showed high sequence similarity to *Azospirillum brasilense* strain and clustered with strains of this species in the phylogenetic tree constructed on the basis of 16S *rRNA* sequence. Isolation of *Azospirillum* strains has been frequently reported from graminaceous plants including maize (Mehnaz and Lazarovits, 2006). The isolates M25 and M28 were identified as *Achromobacter* and *Rhodococcus*, respectively, on the basis of 16S *rRNA* sequence analysis. The isolation of *Achromobacter* has been reported from wheat (Jha and Kumar 2009) and *Prosopis strombulifera* (Sgroy *et al.*, 2009) and that of *Rhodococcus* from potato (Jafra *et al.*, 2006) and *Taxus chinensis* (Wang *et al.*, 2008).

The three *Azospirillum* strains isolated from maize produced higher amount of IAA compared with *Achromobacter* and *Rhodococcus* strains. Azospirilla are considered efficient producers of IAA and have been reported to utilize mainly this plant beneficial trait for growth promotion (Bashan and de-Bashan, 2010) *Azospirillum* strains (ZN1, M1 and M7) produced 23.98, 23.38 and 22.27 mg L^{-1} IAA, respectively. Crozier *et al.* (1988) investigated the production of IAA by *A. brasilense* and indicated that, after 20 h incubation, the liquid media contained more than 20 µg mL^{-1} of IAA. El-Khawas and Adachi (1999) reported that *Azospirillum brasilense* produced the maximum of 46 ppm auxin in 100 µg mL^{-1} tryptophan at 72 h. Different researchers proposed different amounts of IAA produced by different *Azospirillum* strains because of different incubation times, concentrations of precursor i.e., tryptophan and strains used for IAA study. *Achromobacter* strain M25 produced 11.6 mg L^{-1} of IAA in the present study. Jha and Kumar (2009) reported that *Achromobacter* sp. was able to produce 13.5 µg mg^{-1} dry weights IAA. Ma *et al.,* (2009) detected 6.4 mg L^{-1} IAA produced by *Achromobacter* strain. *Rhodococcus* strain M28 produced 11.5 mg L^{-1} in our study but Tsavkelova *et al.* (2005) reported 49.6 µg mL^{-1} IAA production by a strain of this genus using higher amount (200 µg mL^{-1}) of tryptophan as precursor in the growth medium.

The *Azospirillum* strains ZN1, M1 and M7 failed to show halo zone formation on Pikovskaya agar plates and no P-solubilizing activity in Pikovskaya broth was detected by spectrometry. Inability of some *Azospirillum* strains to solubilize phosphates has been reported (Vikram *et al.*, 2007) while others report presence of this trait in *Azospirillum* (Rodriguez *et al.*, 2004). Halo zone formation was observed around colonies of *Achromobacter* strain M25 and *Rhodococcus* strain M28 and both strains were capable of phosphate solubilization in Pikovskaya broth. *Achromobacter* strain M25 was able to solubilize 105.8 mg L^{-1} of phosphorous in Pikovskaya broth. Ma *et al.* (2009) reported 89.6 µg mL^{-1} while Jha and Kumar (2009) reported 31.5 µg mg^{-1} on dry weight basis. Different strains of the same specie differ in their ability to solubilize phosphate. *Rhodococcus* strain M28 solubilized 59 mg L^{-1} of inorganic P. Chen *et al.* (2006) reported P-solubilizing ability of different strains of *Rhodococcus* from 87 to 186.9 mg L^{-1}. This suggest that both the genera *Achromobacter* and *Rhodococcus* are capable of solubilizing inorganic phosphorous but the capacity of solubilization varies among the strains.

During the initial growth stages of plants, cotyledons act as a sole source of nutrients for seedlings (García-Cebrián *et al.*, 2003). In the present study on maize, cotyledons were removed four days after germination to deprive the seedlings of this source of nutrients and to make them more dependent on the applied bacterial inoculants for nutrient supply. The plants from which cotyledons were detached responded more positively to applied inoculum than plants with intact cotyledons. In the controlled-room experiment, the *Azospirillum* strains proved to be more efficient growth promoting strains compared with *Achromobacter* strain M25 and *Rhodococcus* strain M28. This trend was observed in both the seedlings with intact cotyledons as well as seedlings from which cotyledons were detached. In case of intact cotyledon maximum 24% increase in dry weight was observed in plants inoculated with *Azospirillum* strains ZN1 over control. Dry weight in plants with detached cotyledons and inoculated with *Azospirillum* strain ZN1 showed 27% increase over non-inoculated control.

However it was observed that performance of all the inoculated strains was comparable and effect on plant dry weight was not statistically different among the inoculated treatments in which cotyledons were kept intact. In contrast, the inoculated plants from which cotyledons were removed showed a statistical difference among treatments. This indicated that plants became dependent on external nutrient source and dry weight varied depending on the contribution of the inoculated strains. Bacterial strains used in this study belonged to different genera and have shown significant difference in plant growth promoting traits like IAA production and phosphate solubilization in pure cultures. Thus removal of cotyledons clearly differentiated the bacterial strains used as inoculum on the basis of plant growth promotion. Only a few studies related to seed borne diseases and effect on growth of plants have been made on removal of cotyledons (Sonesson, 1994; Alejandro *et al.*, 1995; Kitajima, 2003).

To further investigate the results obtained from controlled-room experiment in which seedlings were harvested at an early growth stage for quick screening of bacterial strains, an experiment on maize was conducted in non-sterilized soil and the inoculated plants were allowed to grow till maturity. As reflected by the plant dry weight, performance of *Azospirillum* strains and *Achromobacter* strain was comparable and *Rhodococcus* strain M28 proved to be less effective. The effect of bacterial inoculants on grain yield was more differentiating as regards the inoculum application and comparable to that observed on plants from which cotyledons were removed for screening of strains under controlled conditions. Inoculation with the strains of *Azospirillum* gave higher grain yield than plants inoculated with *Achromobacter* and *Rhodococcus* as well as non-inoculated control. In this regard sequence of effectiveness as inoculum was ZN1>M7>M1>M25>M28>C and was generally comparable to that observed in case of control-room experiment in which cotyledons were removed. Effect of inoculation of *Azospirillum* on maize has been reported. Mehnaz and Lazarovits (2006) have reported that root dry weight of different maize cultivars inoculated with *Azospirillum brasilense* were 11.5, 5 and 23% more than non-inoculated control and increase in shoot weight was 12.5, 6 and 16% more than control. In another study increase in cob mass (4.6%) due to inoculation with *Azospirillum brasilense* over non-inoculated control was observed (Swędrzyńska, 2000).

The plant growth promoting effect of *Azospirillum* strains observed in the present study may be due to IAA production (de-Bashan *et al.*, 2008) as phosphate solubilization was not detected by any *Azospirillum* strains used in this study. Partial *nifH* was successfully amplified from all the *Azospirillum* strains (results not shown). Therefore nitrogen-fixing activity of inoculated *Azospirillum* strains may also have contributed to plant growth improvement observed in the present study. Increased yield of crop plants have been frequently reported due to inoculation with azospirilla and mostly phytohormone production has been implicated in growth promotion (de-Bashan *et al.*, 2008; El-Katatny, 2010). Phosphate solubilizing strains *Rhodococcus* M28 and *Achromobacter* M25 also increased the grain yield in the present study. Beneficial effects of inoculation with *Achromobacter* have been reported in wheat and maize (Jha and Kumar, 2009; Marques *et al.*, 2010). It has been reported that the *Achromobacter* strain increased root elongation 39% more than control in maize plants. *Rhodococcus* inoculation has also been studied for growth

promotion in plants like pea and orchid (Tsavkelova *et al.*, 2005; Safronova *et al.*, 2006).

In this study detachment of cotyledons at an early growth stage was tested for screening of bacterial isolates for growth promotion of maize under controlled conditions. The detachment of cotyledons proved very effective in short-term experiments as this treatment clearly and effectively differentiated bacterial inocula on the basis of plant growth promoting effects and the results were more comparable to those of pot experiments in which plants were grown to maturity in non-sterilized soil in earthen pots.

***Acknowledgement*:** The research work was supported by a Ph.D. fellowship and PAS/HEC funded project (P.I., Kauser A. Malik), Government of Pakistan.

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