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Isolation and characterization of beneficial indigenous endophytic bacteria for plant growth promoting activity in Molelwane Farm, Mafikeng, South Africa

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Plant-associated bacteria that live inside plant tissues without causing any damage to plants are defined as endophytic bacteria. The present study was carried out to analyze the phenotypic and genotypic diversity of endophytic bacteria associated with *Amaranthus hybridus*, *Solanum lycopersicum* and *Cucurbita maxima*. A total of 50 bacteria were isolated from the roots of the plants. All the isolates were screened for morphological features (Gram reaction, pigmentation, odour, colour, motility and sharp). Isolates exhibiting difference in morphological features were selected for molecular identification. Eight isolates that exhibited differences in phenotypic aspect were subjected to partial 16S-rDNA gene sequencing using polymerase chain reaction (PCR) for phylogenetic analysis. Sequence analysis using Clustal-X version 1.83 software identified the following isolated bacteria: *Stenotrophomonas maltophilia* KC010525, *Pseudomonas putida* KC010526, *P. putida* KC010527, *P. putida* KC010528, *S. maltophilia* KC010529, *Achromobacter xylosoxidans* KC010530, *A. xylosoxidans* KC010531 and *Achromobacte* sp. KC010532. Further evaluation of the bacterial isolates for phosphate solubilization capacity, indole acetic acid (IAA), hydrogen cyanide (HCN) and ammonium gas production, showed all eight bacterial isolates were able to produce IAA (0.32-2.42 mg/mgI⁻¹). However, seven isolates excluding *S. maltophilia* KC010525 showed ability to produce ammonium. HCN was observed in six isolates: *A. xylosoxidans* KC010530, *A. xylosoxidans* KC010531, *A. KC010532*, *P. putida* KC010526, *P. putida* KC010527, and *P. putida* KC010528. When determining the phosphate solubilizing capacity, it was observed that seven solubilized insoluble phosphate in Pikovskya's agar plates produced halo zones (1 to 4 mm). Seven tested bacteria were active against *Fusarium oxysporum*. Therefore, the results indicate that the bacteria isolates may be used as a promising microbial inoculant for plant growth and productivity.

Key words: Plant growth promoting rhizobacteria (PGPR), 16S-rDNA sequencing, HCN production, indole acetic acid (IAA), phosphate solubilization, antifungal activity.

INTRODUCTION

The use of inorganic fertilizers and pesticides for plant protection with sometimes detrimental effect has resulted to the search for environmental friendly sustainable

agricultural practices. To this effect, there is considerable interest in the development of new and alternative control approaches for use in integrated plant growth management

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Abbreviations: PGPR, Plant growth promoting rhizobacteria; HCN, hydrogen cyanide; IAA, indole-3-acetic acid; ACC, 1-aminocyclopropane-1-carboxylate; PBS, phosphate buffered saline; NB, nutrient broth; PCR, polymerase chain reaction; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information

strategies (Compant et al., 2005; Ngoma et al., 2012). In recent years, focus has been on the use of plant growth promoting rhizobacteria (PGPR), as an alternative, environmentally friendly and effective strategy for plant control strategy (Babalola and Glick, 2012; Patel et al., 2012).

Research on plants associated with microorganisms is currently expanding quite rapidly with the identification of new bacterial strains, which are more effective in promoting plant growth (Trivedi and Pandey, 2008; Pereira et al., 2011). PGPR are among the most complex, diverse, and important assemblages in the biosphere (Khan, 2005). They are considered as a group of beneficial free-living soil bacteria for sustainable agriculture and environment (Babalola, 2010). PGPR are characterized by a number of activities, which include the capacity to colonize plant roots surfaces closely adhering to soil interface, increase mineral nutrient solubilization and nitrogen fixation (Khan, 2005; Abou-Shanab et al., 2003).

Also, they have been shown to promote plant growth and yield, suppress plant diseases and soilborne pathogens by the production of hydrogen cyanide (HCN), siderophores, antibiotics, and/or competition for nutrients (bioprotectants) (Kamnev and Lelie, 2000; Abou-Shanab et al., 2003; Idris et al., 2004). Furthermore, PGPR improve plant stress tolerance to drought, salinity, metal toxicity and production of phytohormones such as indole-3-acetic acid (IAA) (biostimulants) (Khan et al., 2009; Verma et al., 2010; Figueiredo et al., 2010).

Lastly, they stimulate the roots of plants to develop and have better establishment during early stages of growth, due to the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase which modulates the level of ethylene by hydrolysing ACC, a precursor of ethylene, in ammonia and α -ketobutyrate (biofertilizers) (Glick et al., 1998).

Numerous PGPR such as: *Burkholderia gladioli*, *Burkholderia cepacia*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Serratia plymuthica*, *Serratia ficaria*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas luteola*, *Erwinia spp*, *Escherichia vulneris*, *Rahnella aquatilis*, *Ewingella americana*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*: *Acinetobacter*, *Proteus penneri*, and *Shigella spp* can be divided into two major groups according to their relationship with the host plants (Khan et al., 2009; Babalola, 2010): (i) extracellular PGPR, existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, and (ii) intracellular PGPR, which exist inside root cells, generally in specialized nodular structures (*Bacillus*, *Pseudomonas*, *Azotobacter*, etc.) (Thakuria et al., 2004; Figueiredo et al., 2010).

In recent years, there are several PGPR such as *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* inoculants currently commercialized that seem to promote growth of plant. However, very little research work has been done on PGPR in the agriculture sector of the North West province Mafikeng. Hence, the

present study was undertaken to explore an alternative approach which can be used as biofertilizers for the control of plant growth without damaging the environment. A series of endophytic bacteria associated to the roots of *Amaranthus hybridus* (smooth amaranth), *Solanum lycopersicum* (Tomato) and *Cucurbita maxima* (Pumpkin) were isolated and identified using morphological, biochemical and molecular approaches. These native bacteria were also assessed for their plant growth promoting activities such as: IAA, HCN and ammonia production. These findings could be incorporated in plant growth management strategies.

MATERIALS AND METHODS

Field sites and sample collection

This study was carried out at Molelwane Farm, North West province, South Africa. Molelwane Farm is located out of Mafikeng City between 25 and 28° south of the equator and 22 and 28° longitude east of the Greenwich meridian. The farm shares an international border with the Republic of Botswana in the North and 260 km West of Johannesburg. It is built on the open veld at an elevation of 1.500 m, by the banks of the Upper Molopo River. Climatic conditions vary significantly from West to East. The Western region receives less than 300 mm per annum, the Central region around 550 mm p.a., while the Eastern and South-Eastern region receives over 600 mm per annum (De Villiers and Mangold, 2012).

Sterile techniques were used during each collection. Each sample was labelled immediately and placed on a dry cool place to avoid moisture accumulation or excessive drying. The root of *A. hybridus*, *S. lycopersicum* and *C. maxima* plants were collected at different sites of Molelwane Farm, in March and April. Intact root systems for each plant were dug out and carefully taken immediately to the laboratory in sterile polyethylene bags and stored at 4°C. A total of three root systems samples were collected for the isolation of endophytic bacteria.

Isolation of culturable endophytic bacteria

To isolate PGPR from the samples, the roots were first soaked in 70% ethanol for 5 min, in 6.25% sodium hypochlorite for 10 min, followed by several rinses in sterile distilled water. Plant materials were then suspended in 0.05 M phosphate buffered saline (PBS) and mashed with a sterilized mortar and pestle. The obtained root suspensions were serially diluted ten-fold using PBS prior to spreading on plate count agar (Sigma Aldrich, South Africa) (Piromyou et al., 2010). Aliquots of each dilution (0.5 ml) were plated on solidified Cetrinide, MacConkey and Aeromonas agar (Sigma Aldrich, South Africa). All plates were incubated for 24 h at 37°C. Suspect colonies showing morphological difference were selected and streaked plated on freshly prepared media to obtain pure colonies. All the isolates were preserved at 4°C on equal volumes of nutrient agar and 30% glycerol (Kumar et al., 2012).

Biochemical characterization of suspect endophytic bacteria

All selected bacteria were further characterized by Gram's staining and biochemical tests. The various tests performed were: Catalase activity, oxidase activity, citrate test, indole test, methyl red test, voges-proskauer test, motility test, triple sugar ion test, nitrate reduction test (Krieg and Hoff, 1984).

Indole acetic acid (IAA) production

A modified colorimetric estimation procedure was used for determination of IAA as described by Patten and Glick (2002); Patel et al. (2012). 500 µl of endosphere bacteria strains obtained earlier from *A. hybridus*, *S. lycopersicum* and *C. maxima* were grown in 50 ml of nutrient broth (NB) containing 0.1% DL tryptophan and incubated at 28°C on a rotator shaker (SI-600, LAB Companion, Korea) for 48 h. A 5 ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min. 2 ml culture supernatant was transferred to a fresh tube and mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid and 1 ml 0.5 M FeCl₃ solution). The mixture was then incubated at room temperature for 25 min, and the development of pink colour indicates IAA production, the absorbance of positive reaction was read at 530 nM in a spectrophotometer. The colours produced by the respective strains were categorized into low, medium and high. The experiment was performed thrice with three replicates for each bacterial strain.

Hydrogen cyanide production

Screening of bacterial isolates for HCN production was done using Castric's method (Castric, 1975). All suspected isolates were grown in 10% tryptone soy agar supplemented with glycine (4.4 g l⁻¹) (Sigma, South Africa). A Whatman filter paper No. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution was placed to the underside of the Petri dish lids. To avoid the escape of the gas, the plates were sealed with parafilm and incubated at 30°C for 5 days and the production of HCN was determined by the change in colour of filter paper from yellow to red-brown.

Phosphate solubilization

Phosphate solubilization activities were assayed by spotting 10 µl of cultures separately on the top of Pikovskya's agar plates and incubating at 30°C for one week as described by Nautiyal (1999). The colonies showing the clear halo zone around them indicated solubilization of mineral phosphate. The halo size produced by the respective strain was measured and categorized into low, medium and high on the basis of zone diameter.

Production of ammonia

Bacterial isolates were tested for the production of ammonia as follows: freshly grown bacterial cultures were inoculated in 10 ml nutrient broth and incubated at 30°C for 48 h in a rotator shaker (SI-600, LAB Companion, Korea). After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of a yellow to brown colour indicated a positive reaction for ammonia production (Cappuccino and Sherman, 1992).

Antifungal activity

Overnight grown bacterial cultures were streaked on four ends of the plate into potato dextrose agar and spores of fungal species *Fusarium oxysporum* were placed in the middle of the plates and incubated at 28°C for 5 days. The percent of radial growth inhibition were recorded by the following formula:

$$\text{PIRG} = (R1 - R2 / R1) \times 100$$

Where, R1, Radial growth of *F. oxysporum* in control plate; R2, radial growth of *F. oxysporum* interacting with antagonistic bacteria

(Noori and Saud, 2012).

Molecular identification of endophytic colonizing bacteria

Molecular identification has provided powerful tools to investigate microbial community in the world at species level. This technique is very important since it provides informative insight about the bacteria, possible kind of bioactive compounds, and if it is novel or not (Donate-Correa et al., 2004). The identification of the bacterial isolates in the present study was based on 16S-rDNA gene sequence analysis.

Extraction of genomic DNA

Total genomic DNA of cultivated isolates was purified following Zymo-Research Fungal/Bacterial DNA kit. Colonies were suspended in 1 ml sterile distilled water in eppendorf tubes and centrifuged at 10,000 rpm for 5 min and the supernatant discarded. Briefly, pellets were suspended in 750 µl lysis solution and vortexed at 14000 rpm for 5 min, followed by centrifugation at 10 000 rpm for 1 min. 400 µl of the upper aqueous phase was aliquoted into a new eppendoff tube and centrifuged at 7000 rpm for 1 min. 1200 µl of buffer was added to the filtrate and 800 µl of the mixture was transferred to the new collection tube and centrifuged at 10,000 rpm for 1 min. The filtered DNA was pre washed by adding 200 µl DNA pre-wash buffer and centrifuged at 10,000 rpm for 1 min. 500 µl of DNA wash buffer was added to the new collection tube and centrifuged at 10,000 rpm for 1 min. Finally, 100 µl of DNA elution buffer was added to elute the DNA in a clean 1.5 ml micro-centrifuge tube. The concentration of purification of DNA was assessed using a nanodrop (Bio-Rad, South Africa).

Polymerase chain reaction (PCR) amplification

The 16S rDNA nucleotide sequences were determined by PCR using Engine DYAD Peltier thermal cycler (BioRad, USA). Reaction volume of 50 µl, containing: 25 µl PCR Master Mix, 2 µl template DNA, 19 µl nuclease free water and 2 µl of each oligonucleotide primer was prepared as reported by Ntougias et al. (2004). The forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse 1492R (5'-TGA CTG ACT GAG GCT ACC TG-3') were commercially synthesised by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Thermal cycling conditions were as follows: one cycle of 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 5 min, followed by final cycle of extension of 72°C for 7 min and incubated at 4°C forever. Amplified PCR products were resolved in 1% agarose gel stained with ethidium bromide (10 µg ml⁻¹) and visualised with Syngene Ingenius Bioimager (UK) to confirm the expected size of the product. The 16S rDNA sequences of the isolated were deposited in GenBank database for accession numbers.

Nucleotide sequence determination

PCR products of the 16S rDNA of the strains were analyzed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. The homology of partial sequences obtained were compared with the sequences from the DNA databases and similarity showing above 95% were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST

Table 1. Morphological characteristic of odourless, motile, Gram negative rods bacterial isolates in endophytic roots at Molelwane farm.

Isolate	Pigmentation	Root variety in the field	Colour
TEC59	Pigmented	<i>Amaranthus hybridus</i>	Yellow
TEA61	Pigmented	<i>Amaranthus hybridus</i>	Yellow
TEA53	Pigmented	<i>Amaranthus hybridus</i>	Yellow
PM22	Pigmented	<i>Cucurbita maxima</i>	Dark red
TOM75	None	<i>Solanum lycopersicum</i>	Light red
TOC68	None	<i>Solanum lycopersicum</i>	Light red
API69	None	<i>Cucurbita maxima</i>	Light red
TEM56	Pigmented	<i>Amaranthus hybridus</i>	Red

server (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990).

Phylogenetic analysis

All confirmed sequencing results obtained in this study was analyzed and edited using Bio-Edit softwares (Hall, 1999). The 16S rDNA sequence of the strains was used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions. To identify putative close phylogenetic relatives. Multiple sequence alignments were obtained using Clustal-X version 1.83 software (Thompson et al., 1997) against corresponding nucleotide sequences retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969). Phylogenetic analysis was performed using the program MEGA version 5.10 (Tamura et al., 2011) and neighbour joining (Saitou and Nei, 1987), maximum-parsimony (Rzhetsky and Nei, 1992), maximum likelihood analyses were obtained for each gene (Fitch, 1986) trees was constructed. The methods were used in order to expound on the phylogeny and for better comparison. Bootstrap analyzes were performed using 1000 replications for neighbour joining, maximum-parsimony, maximum likelihood. The sequences were checked for putative chimeric artifacts using the Chimera-Buster program. No chimeric sequences were compared to the closest relatives in the NCBI Gen- Bank database by BLAST program. Manipulation and tree editing were carried out using Tree View (Page, 1996).

RESULTS

In this work, from the 50 bacteria cultured on plates, only eight isolated strains were suspected to be PGPR. These bacteria were confirmed based on their different morphological characteristics (Table 1). They were designated as TEM56, TEC59, TEA61, TEA53, PM22, TOM75, TOC68 and API69. These isolated bacteria were further characterised by Gram staining and biochemical test. As shown in Tables 1 and 2, the biochemical and morphological observations of the isolated bacteria showed that most of them were fast growers; all their cells were Gram negative, rod shaped and motile in reaction. They differed in colour according to media used (Cemetride, McConkey and/or Aeromonas agar), and all were odourless. Among them, five (TEM56, TEC59, TEA61, TEA53 and PM22) produced pigments on plates: the isolates TEM56 and PM22 produced pigment on MacConkey while TEC59,

TEA61, and TEA53 on Cetrimede agar. Their diameter varied from 0.2 to 2 mm.

Identification and phylogenetic analysis of isolates

All bacteria which had been classified by biochemical and morphological assay were analyzed by PCR profile to confirm their strain identification as shown in Figure 1. Eight unique band positions were identified on UV and sequenced by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. The 16S-rDNA sequences were then deposited in GenBank and assigned accession numbers indicated in parentheses: TOM 75 (KC010530), TOC 68 (KC010531) API 69 (KC010532) TEM 56 (KC010525) TEC 59 (KC010529) TEA 61 (KC010526) TEA 53 (KC010527) PM 22 (KC010528). Results of their closest relatives are shown in Table 3 and in Figure 2 the phylogenetic tree shows a better picture of the relationships among them. The endophytic bacteria TEM56 and PM22 displayed close homology to *Stenotrophomonas maltophilia* while the group of bacteria TEC59, TEA61, TEA53 displayed close relationship with *P. putida*. The isolates TOM75, TOC68 and API69 to *Achromobacter xylosoxidans* as shown in Table 4. The identified isolates of *S. maltophilia* (KC010525), *P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528) *S. maltophilia* (KC010529), *A. xylosoxidans* (KC010530), *A. xylosoxidans* (KC010531) and *Achromobacter* sp. (KC010532) were analyzed for their plant growth promoting activities such as, phosphate solubilization, IAA production, HCN production, and antifungal activity against *F. oxysporum*. Production of HCN was detected in six isolates (Figure 3). It showed that out of eight isolated bacteria screened for phosphate solubilization on modified Pikovskya's agar plates, seven isolates; *A. xylosoxidans* (KC010530), *A. xylosoxidans* (KC010531), *Achromobacter* (KC010532), *P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528) showed the development of sharp phosphate solubilization zones, ranging from 1 to 4 mm while the isolate *S. maltophilia* (KC010525) was negative (Figure 4). The IAA data showed that, all eight bacterial isolates were able to produce IAA but their concentration was

Table 2. Biochemical characterization of bacterial endophytes from native *Amaranthus hybridus*, *Cucurbita maxima* and *Solanum lycopersicum*.

Isolate	Catalase	Oxidase	Citrate	Methyl red	Voges- Proskauer	Nitrate reduction	H ₂ S production
TEC 59	+	+	+	-	-	-	-
TEA 61	+	+	+	-	-	-	-
TEA 53	+	+	+	-	-	-	-
PM 22	+	-	+	-	-	+	-
TOM 75	+	+	+	-	-	+	-
TOC 68	+	+	+	-	-	+	-
API 69	+	+	+	-	-	+	-
TEM 56	+	-	+	-	-	+	-

+, Activity; -, no activity.

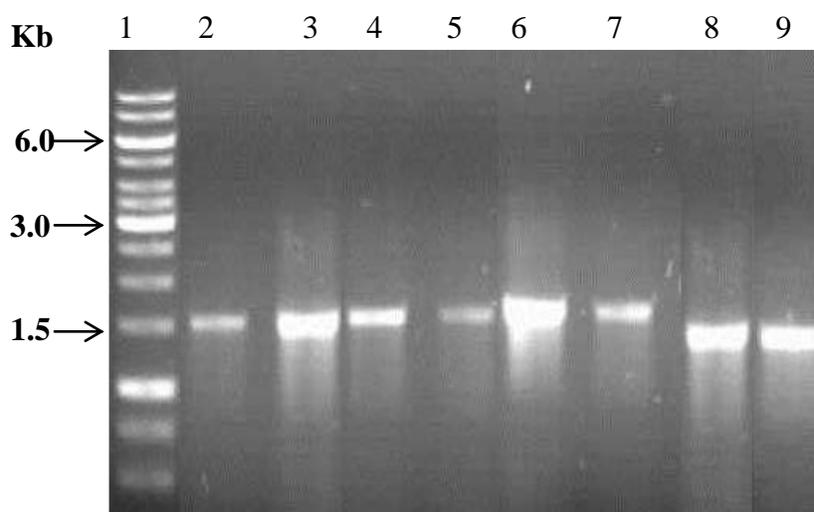


Figure 1. PCR profiles of 16S rDNA fragments amplified from bacteria isolated from the root of *Amaranthus hybridus*, *Solanum lycopersicum* and *Cucurbita maxima*: from left to right 1 Kb marker (1), TEM56 (2), PM22 (3), TEC59 (4), TEA61 (5), TEA53 (6), TOM75 (7), TOC68 (8), API69 (9).

very low ranging between 0.162 to 0.324 mg/ml (Table 4). Ammonia production is another important trait of PGPR that indirectly influence plant development. Seven bacterial isolates were able to produce ammonia while one *S. maltophilia* KC010525 was unable to do so. The number of positive and negative bacterial isolates for each plant growth promoting activity was observed and given in Table 4. For determination of antifungal activity of *F. oxysporum*, results show that seven bacterial isolates (except for *S. maltophilia* (KC010525)) exhibited antifungal activity against *F. oxysporum* pathogen in culture assay, whereas isolates *P. putida* (KC010526), *P. putida* (KC010527) and *P. putida* (KC010528), showed the maximum percent inhibition rate of 65, 63 and 63%, respectively. The inhibition assay using *Achromobacter* sp (KC010532) revealed less reduction in radial growth compared to *pseudomonas* sp. Their inhibition rates are: *A. xylosoxidans* (KC010530) 62%, *A. xylosoxidans*

(KC010531) 60%, *Achromobacter* (KC010532) 58% while *S. maltophilia* (KC010529) has an inhibition zone of 55% (Figure 5 and Table 4).

DISCUSSION

The bacterial community from the root of three plants, *A. hybridus*, *S. lycopersicum* and *C. maxima*, were assessed using a combination of morphological, bio-chemical, including molecular tools and plant growth approaches. The identification of bacteria was further confirmed at phylogenetic level. The Gram negative rod shaped morphology, negative for oxidase and catalase test of TEC 59, TEA 61 and TEA 53 qualified them to be members of *Pseudomonas* sp. Similarly, Gram negative, rod shaped morphology, negative to oxidase, positive to catalase as well as to citrate are attributes of PM 22 and TEM 56 denominated to be members of *Stenotrophomas*.

Table 3. Results of 16S rDNA sequence similarities of endophytic bacteria isolated and GenBank accession numbers using BLASTN Algorithm.

Isolate code	Sequence length (bp)	Closest related in database	Accession number	Similarity (%)	E-value
TOM 75	1391	<i>A. xylosoxidans</i>	GQ 415969.1	99	0
TOC 68	1379	<i>A. xylosoxidans</i>	GQ 415969.1	99	0
API 69	1363	<i>Achromobacter</i>	GQ.415969.1	99	0
TEM 56	1380	<i>S. maltophilia</i>	JQ 619623	99	0
TEC 59	1343	<i>S.maltophilia</i>	JQ 619623	100	0
TEA 61	1353	<i>P. putida</i>	JX 569146.1	99	0
TEA 53	1349	<i>P. putida</i>	JX 569146.1	99	0
PM 22	1277	<i>P. putida</i>	JX 569146.1	99	0

In addition, TOM 75, TOC 68 and API 69 were closely related to *Achromobacter*. Furthermore, the clustering of all the bacteria was supported by 100, 60, 94 and 100% bootstrap. The similarity level of TEM56 and TEC59 species with *S. maltophilia* JQ 619623 lied between 99 to 100%. The isolated bacteria TOM75 and TOC68 showed 99% similarity with *A. xylosoxidans* GQ 415969.1 while TEA61, TEA53 and PM22 showed 99% similarity with *P. putida* JX 569146.1. The Genbank database identified the isolates bacterial as *P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528), *S. maltophilia* (KC010529), *S. maltophilia* (KC010525), *A. xylosoxidans* (KC010530), *A. xylosoxidans* (KC010531) and *Achromobacter* sp. (KC010532).

The identified isolates were screened *in vitro* for their plant growth promoting activities. The most common, best characterized and physiologically most active auxin in plant is IAA. All the isolates tested produced IAA and consequently, are considered as IAA producing rhizobacteria. Recent studies have shown that IAA biosynthesis is greatly influenced by L-TRP precursor. L-TRP is believed to be the primary precursor for the formation of IAA, in several microorganisms (Yasmin et al., 2009; Ahmad and Khan, 2011). This phytohormone affects many physiological activities of plant such as cell enlargement, cell division, root initiation, and growth rate. However, it may also acts as a signalling molecule during the onset of symbiosis (Patten and Glick, 1996).

In this study, the range of IAA production was very low (0.32 to 2.42 mg l⁻¹) as compared to those presented in other reports. Ahmad et al. (2008) reported levels of 2.13 and 3.6 mg l⁻¹ for *Pseudomonas* species, whereas Gravel et al. (2007) reported levels of 3.3 and 6.2 mg l⁻¹ for *P. putida*. The results show that *Pseudomonas* strains produced IAA above 1.0 mg ml⁻¹, with the highest amount of 2.42 mg ml⁻¹ found in the culture medium from *P. putida* strain. The other five strains yielded lower amounts at rates of 0.32 to 1.12 mg ml⁻¹. Kumar et al. (2012) revealed in this study, that IAA production by PGPR could vary among different species and strains of rhizobacteria, culture and medium conditions. The study further shows that IAA production could also be influenced

by growth stage and substrate availability. These findings are further strengthened by the low levels of IAA obtained in this study (Table 4). It has been reported that a low level of IAA produced by rhizobacteria promotes primary root elongation, whereas a high level increases lateral and adventitious root formation but inhibits the primary root growth (Xie et al., 1996). Such type of clarification suggested that even at low concentration, these isolates may be able to stimulate the development of the plant. Seven bacterial isolates: *P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528), *S. maltophilia* (KC010529), *A. xylosoxidans* (KC010530), *A. xylosoxidans* (KC010531) and *Achromobacter* sp. (KC010532) showed the ability to solubilize complex calcium phosphate while *S. maltophilia* (KC010525) was unable to do so as the halo zone was not significantly different from the control. Similar research reports have been documented by Rodríguez et al. (2006) that bacterial strains belonging to these genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, and *Erwinia* have the ability to solubilize insoluble inorganic phosphate (mineral phosphate) compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite, and rock phosphate. Solubilization of insoluble organic phosphate has been attributed to the availability of optimum pH required for growth and the limitation of nutrients (Kumar et al., 2010). In this study, the result show that majority of the PGPR do have phosphate solubilizing activity and such bacteria as suggested by Kumar et al. (2010) play an important role in making available solubilised fraction of various phosphate minerals in soil for plant growth. Another important trait of PGPR is the production of HCN which plays an important role in the biological control of several soil-borne pathogenic fungi (Ramette et al., 2003). The study revealed that six bacterial isolates with the exception of *S. maltophilia* KC010525 and *S. maltophilia* (KC010529) were positive for HCN. However, the degree of production ranged from moderate (*P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528)) to weak (*A. xylosoxidans* (KC010530), *A. xylosoxidans* (KC010531))

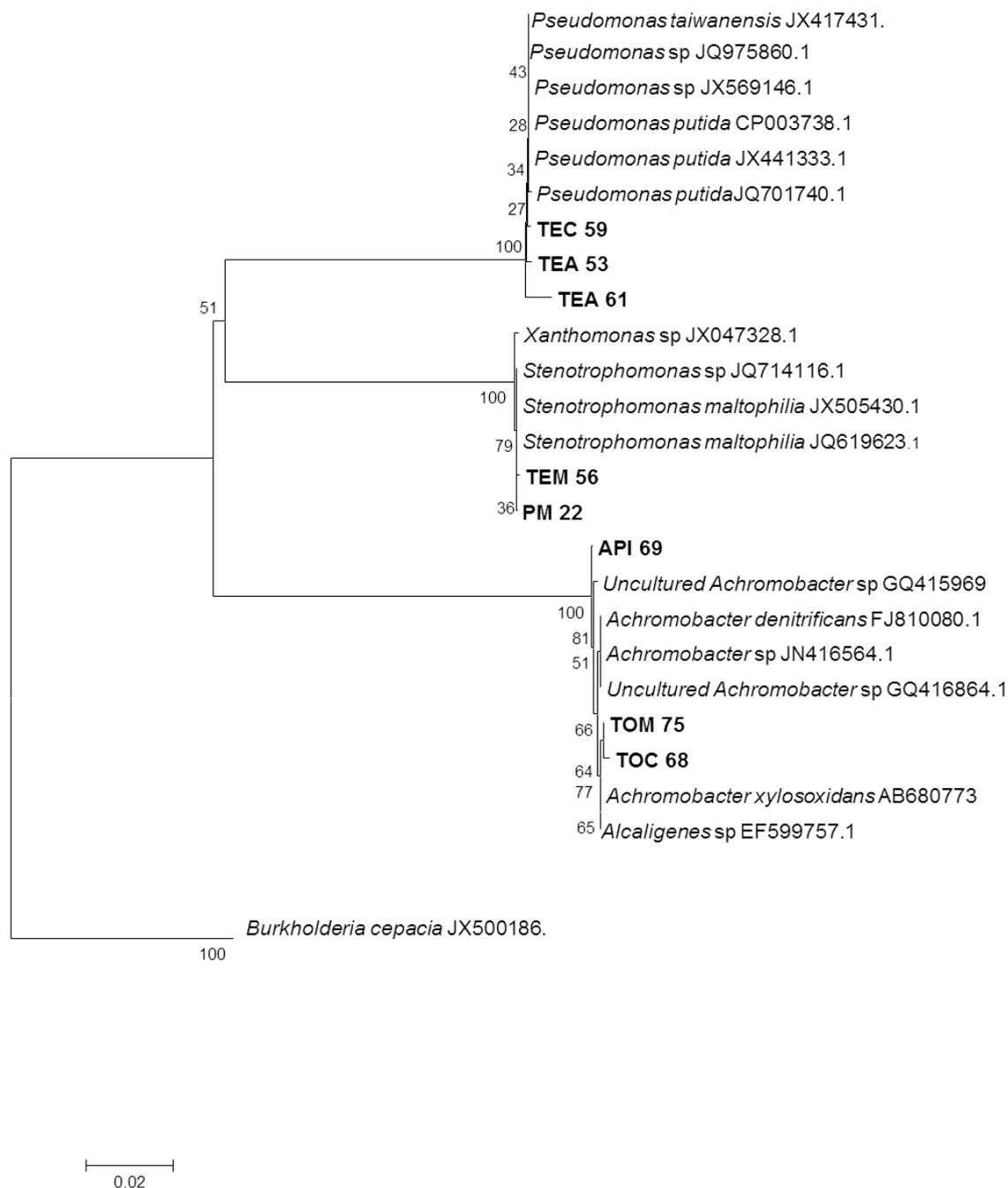


Figure 2. Phylogenetic tree expressing the relationships of identified bacterial endophytes (bold font) to taxonomically similar bacteria based on the 16S rDNA sequences. The tree was clustered with the neighbour-joining method using MEGA 4.1 package. Bootstrap values based on 1000 replications were listed as percentages at the nodes. The scale bar indicates 0.05 substitutions per nucleotide position. The GenBank accession number is given in parentheses for each organism. The 16S rDNA sequence of *Burkholderia cepacia* JX500186 was utilized as an out group.

and *Achromobacter* sp. (KC010532) as compared to the control. It is also worth noting that similar results were obtained with other *P. fluorescens*, *P. aeruginosa* and *Chromobacterium violaceum* (Seddiqui et al., 2003).

Several *Rhizobium* spp were also established as HCN producers by Antoun et al. (1998). In the present study, only *S. maltophilia* (KC010525) isolate showed negative results for ammonia production (Table 4). It has been

Table 4. Assessment of plant growth promoting traits and antifungal activities of selected bacteria isolates.

Sample	IAA Production (mg/ml ⁻¹)	HCN production	Phosphate solubilisation (mm)	Ammonium production	Antifungal activities (%)
<i>A. xylosoxidans</i> (KC010530)	+ (0.77)	++	+ (2)	+	+ (62%)
<i>A. xylosoxidans</i> KC010531	+ (0.57)	++	+ (2)	+	+ (60%)
<i>Achromobacter</i> (KC010532)	+ (0.56)	++	+ (2)	+	+ (58%)
<i>S. maltophilia</i> (KC010525)	+ (0.32)	-	-	-	-
<i>S. maltophilia</i> (KC010529)	+ (0.49)	-	+ (1)	+	+ (55%)
<i>P. putida</i> (KC010526)	+ (2.42)	+++	++ (2)	+	+ 65%
<i>P. putida</i> (KC010527)	+ (1.12)	+++	++ (3)	+	+ (63%)
<i>P. putida</i> (KC010528)	+ (1.07)	+++	+++ (4)	+	+ (63%)

+, Activity; -, no activity

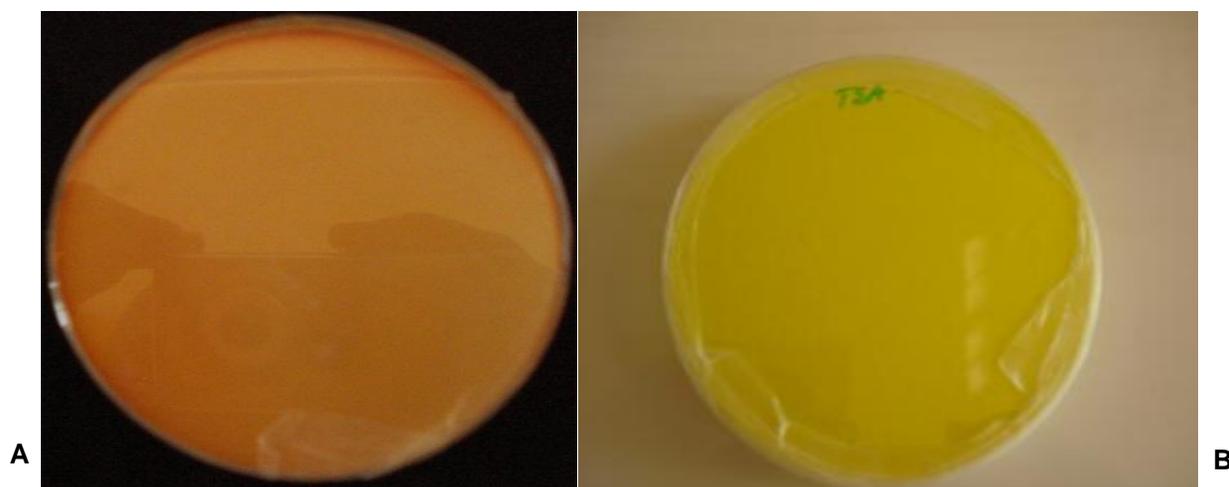


Figure 3. Colour change of filter papers (A) compare to control (B) indicating the detection of HCN production.

assumed that inoculation with such bacteria may enhance the plant growth as a result of their ability to fix Nitrogen (N₂) to Ammonia (NH₃) making it an available nutrient for plant growth (Hayat et al., 2010). The general concept of the success of PGPR was attributed to the inhibitory effects of antagonistic organisms (Yildiz et al., 2012).

All eight bacterial isolates excluding *S. maltophilia* (KC010525) were found to be antagonistic at varying degree to the soil borne fungal pathogens (*F. oxysporum*) on dextrose plate agar. The inhibition process observed *in vitro* may suggest the secretion of fungicidal metabolites by the bacteria (Figueiredo et al., 2010). However, the degree of inhibition ranged from 63%, for *Pseudomonas*, 60% for *Achromobacter* and 55% for *Stenotrophomas*. Similarly, Tziros et al. (2007) reported that bacterial isolate especially *Pseudomonas* was effectively used as biological control of *F. wilt* in watermelon especially at the early stage of plant and disease development. Other studies showed the beneficial effect of PGPR to control *F. oxysporum* in tomato (Yigit and Dikilitas, 2007) and to

enhance suppression of *F. oxysporum* of radish (De Boer et al, 1999).

Conclusions

The results indicate that all endophytic bacterial isolates from *A. hybridus*, *S. lycopersicum* and *C. maxima* plants, were found to be similar to *Pseudomonas*, *Stenotrophomas*, *Achromobacter* after phylogenetic analysis. These isolates produced at least one of the characteristics such as IAA, HCN, phosphate solubilisation, ammonia and antifungal activity that may benefit the plant for its development and nutrition. In particular, bacteria such as *P. putida* (KC010526), *P. putida* (KC010527), *P. putida* (KC010528) were found to be the most efficient PGPR which solubilised insoluble phosphorus, produced IAA, ammonia, HCN and showed antifungal activity followed by *Achromobacter*, considered as an important plant growth promoting rhizobacteria. These isolates might have the potential to be used as inoculant for plant growth development. However, the good results obtained

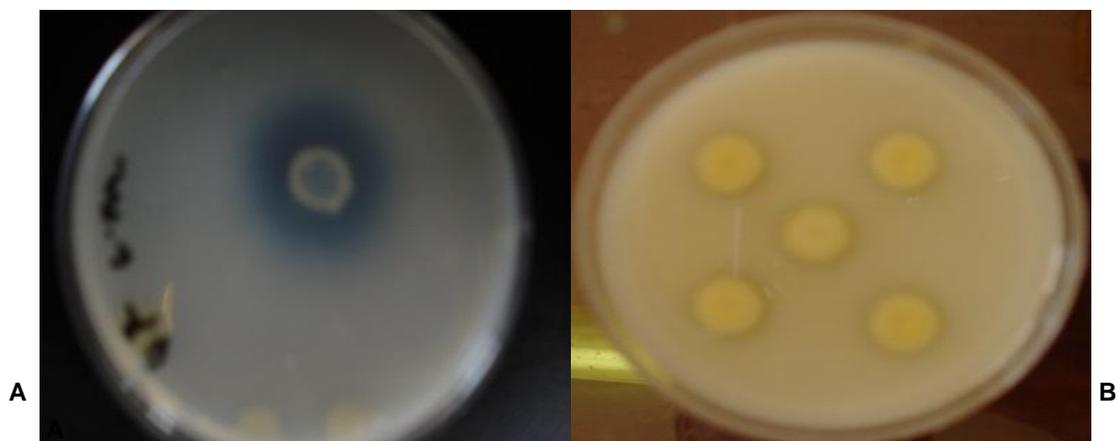


Figure 4. Halo formation on Pikovskya's agar plates (A) as compared to the control (B).



Figure 5. Bacterial antifungal activity in the potato dextrose agar. *F. oxysporum* plug growth was completely inhibited in the presence of the endophytic bacteria streaked in the plates (A), as compared to the control (B), which had no bacteria.

in vitro cannot always be dependably reproduced under field conditions. As a result, further research on field based trial using these bacteria obtained in this study would be necessary for understanding their potential in agro-ecosystem as PGPR.

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REFERENCES

- Abou-Shanab RI, Angle JS, Delorme TA, Chaney RL, vanBerkum P, Moawad H, Ghanem K, Ghazlan HA (2003). Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. *New Phytologist*. 158: 219-224.
- Ahmad F, Ahmad I, Khan MS (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth-promoting activities. *Microbiol. Res.* 163: 173-181.
- Ahmad F, Khan MS (2011). Assessment of plant growth promoting activities Rhizobacterium *Pseudomonas putida* under insecticide-stress. *Microbiol. J.* 1(2): 54-64.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Antoun H, Beauchamp CJ, Goussard N, Chabot R, Lalonde R (1998). Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus*. L). *Plant Soil.* 204:57.
- Babalola OO (2010). Beneficial bacteria of agriculture importance. *Biotechnol. Lett.* 32:1559-1570.
- Babalola OO, Glick BR (2012). Indigenous African agriculture and plant associated microbes: Current practice and future transgenic prospects. *Sci. Res. Essays* 7(28):2431-2439.
- Cappuccino JG, Sherman N (1992). Biochemical activities of microorganisms. In: *Microbiology, a laboratory manual*. The Benjamin / Cummings publishing Co. California, USA.
- Castric PA (1975). Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 21:613-618.
- Compant S, Duffy B, Nowak J, Clement C, Ait Barka E (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. *Appl. Environ. Microbiol.* 71(9): 4951-4959.

- De Villiers B, Mangold S (2012). The biophysical environment. Geography Department, Potchefstroom University, Potchefstroom North West Dept. Agriculture, Conservation and Environment. Mafikeng.
- De Boer M, van der Sluis L, van Loon LC, Bakker PAHM (1999). Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of *Fusarium* wilt of radish. *European J. Plant Pathol.* 105: 201-210.
- Donate-Correa J, Leon-Barrios M, Perez-Galdona R (2004). Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree-shrub legume endemic to the Canary Island. *Plant Soil.* 266:261-272.
- Fitch WM (1986). An estimation of the number of invariable sites is necessary for the accurate estimation of the number of nucleotide substitutions since a common ancestor. *Prog. Clin. Biol. Res.* 218: 149-159.
- Figueiredo MVB, Seldin L, Araujo FF, Mariano RLM (2010). Plant growth promoting rhizobacteria: Fundamentals and applications. *Plant growth and health promoting bacteria.* DOI 10.1007/978-3-642-13612-2-2.
- Glick BR, Penrose DM, Li J (1998). A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. *J. Theor. Biol.* 190:63-68.
- Gravel V, Antoun H, Tweddell RJ (2007). Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). *Soil Biol. Biochem.* 39:1968-1977.
- Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95-98.
- Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* DOI 10.1007/s13213-010-0117-1.
- Jukes TH, Cantor CR (1969). Evolution of protein molecules: Mammalian Protein Metabolism. (3rd edn), Academic Press, New York.
- Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004). Bacterial communities associated with flowering plants on the Ni hyper accumulator *Thlaspi goesingense*. *Appl. Environ. Microbiol.* 70:2667-2677.
- Kamnev AA, Lelie D (2000). Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. *Biosci. Rep.* 20:239-258.
- Khan, AG (2005). Role of soil microbes in the rhizosphere of plants growing on trace metal contaminated soils in phytoremediation. *J. Trace Elem. Med. Biol.* 18:355-364.
- Khan MS, Zaidi A, Wani PA, Oves M (2009). Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environ. Chem. Lett.* 7:1-19.
- Kreig NR, Holf JG (1984). *Bergeys Manual of Systematic Bacteriology.* William and Wilkins, Baltimore, USA.
- Kumar A, Bhargava P, Rai LC (2010). Isolation and molecular characterization of phosphate solubilizing *Enterobacter* and *Exiguobacterium* species from paddy fields of Eastern Uttar Pradesh, India. *Afric. J. Microbiol. Res.* 4(9) 820-829.
- Kumar A, Kumar A, Devi S, Patil S, Payal C, Negi S (2012). Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Res. Sci. Technol.* 4(1):01-05.
- Nautiyal CS (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS. Microbiol. Lett.* 170: 265-270.
- Ngoma L, Babalola OO, Ahmad F (2012). Ecophysiology of plant growth promoting bacteria. *Sci. Res. Essays.* 7(47): 4003-4013.
- Noori MSS, Saud HM (2012). Potential Plant Growth-Promoting Activity of *Pseudomonas* sp isolated from Paddy soil in Malaysia as biocontrol agent. *J. Plant Pathol. Microb.* 3:120. doi:10.4172/2157-7471.1000120.
- Ntougias S, Zervakis GI, Kavroulakis N, Ehaliotis C, Papadopoulou KK (2004). Bacterial diversity in spent mushroom compost assessed by amplified rDNA restriction analysis and sequencing of cultivated isolates. *Syst. Appl. Microbiol.* 27:746-754.
- Page RD (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.
- Patel HA, Patel RK, Khristi SM, Parikh K, Rajendran G (2012). Isolation and characterization of bacterial endophytes from *Lycopersicon esculentum* plant and their plant growth promoting characteristics. *Nepal J. Biotechnol.* 2(1): 37-52.
- Patten C, Glick B (2002). Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* 3795-3801.
- Pereira P, Ibanez F, Rosenblueth M, Etcheverry M, Martinez-Romero E (2011). Analysis of the bacterial diversity associated with the roots of maize (*Zea mays* L.) through culture-dependent and culture-independent Methods. *Intl. Scholarly Res. Network Ecol.* doi:10.5402/2011/938546.
- Piromyong P, Buranabanyat B, Tantasawat P, Tittabutr P, Boonkerd N, Teamroong N (2010). Effect of plant growth promoting rhizobacteria (PGPR) inoculation on microbial community structure in rhizosphere of forage corn cultivated in Thailand. *Eur. J. Soil Biol.* 47:44-54.
- Ramette A, Moëne LY, Défago G (2003). Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS. Microbiol. Ecol.* 44: 35-43.
- Rodriguez H, Fraga R, Gonzalez T, Bashan T (2006). Genetic of phosphate solubilisation and its potential applications for improving plant growth-promoting bacteria. *Plant Soil.* 287:15-21.
- Rzhetsky A, Nei M (1992). A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.* 9: 945-967.
- Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731-2739.
- Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro RC, Khan MR (2004). Characterization and screening of bacteria from the rhizosphere of rice grown in acidic soils of Assam. *Curr. Sci.* 86: 978-985.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acid. Res.* 25: 4876-4882.
- Trivedi P, Pandey A (2008). Recovery of plant growth-promoting rhizobacteria from sodium alginate beads after 3 years following storage at 4°C. *J. Ind. Microbiol. Biotechnol.* 35:205-2.
- Tziros G, Lagopodi AL, Tzavella-klonari K (2007). Reduction of *Fusarium* wilt in watermelon by *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* WCS365. *Phytopathol. Mediterr.* 46:320-323.
- Verma JP, Yadav J, Tiwari KN, Lavakush, Singh V (2010). Impact of plant growth promoting rhizobacteria on crop production. *Intl. J. Agric. Res.* 5 (11): 954-983.
- Whiting SN, deSouza MP, Terry N (2001). Rhizosphere bacteria mobilize Zn for hyper accumulation by *Thlaspi caerulescens*. *Environ. Sci. Technol.* 15:3144-3150.
- Xie H, Pasternak JJ, Glick BR (1996). Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that over produce indole acetic acid. *Curr. Microbiol.* 32:67-71.
- Yasmin F, Othman R, Sijam K, Saad SM (2009). Characterization of beneficial properties of plant growth-promoting rhizobacteria isolated from sweet potato rhizosphere. *Afric. J. Microbiol. Res.* 3(11): 815-821.
- Yigit F, Dikilitas (2007). Control of *Fusarium* wilt of tomato by combination of *Fluorescent Pseudomonas*, non-pathogen *Fusarium* and *Trichoderma harzianum* T-22 in Greenhouse conditions. *Plant Pathol. J.* 6(2): 159-163.
- Yildiz HN, Handan HA, Dikilitas M (2012). Screening of rhizobacteria against *Fusarium oxysporum* f. sp. *melongenae*, the causal agent of wilt disease of eggplant. *Afr. J. Microbiol. Res.* 6(15):3700-3706.