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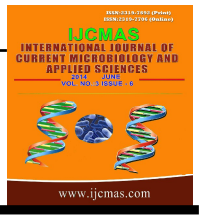

Original Research Article

Screening and Characterization of Plant Growth and Health Promoting Rhizobacteria

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Hydrogen cyanide, multiple PGPR activities, Heavy metal tolerance, Indole-acetic acid.

A B S T R A C T

Rhizobacteria possessing multiple plant growth promoting activities were isolated from the rhizospheric soils of plants growing in semi arid region. Plant Growth Promoting Rhizobacterial (PGPR) strains were isolated and screened for their plant growth promoting activities like phosphate solubilization, production of indole-acetic acid, ammonia, hydrogen cyanide (HCN), catalase, amylase, cellulase, and chitinase. All the isolates solubilized phosphate, 33% of the isolates produced ammonia, 27% produced lipase, 53% produced amylase, 50% chitinase, 17% chitinase and 40% produced indole-acetic acid and a single isolate produced HCN. Four isolates having multiple plant growth promoting activities were selected and identified on the basis of 16S rDNA sequence. These isolates showed heavy metal tolerance; up to 400 µg/ml Pb and Cr, 50 µg/ml Co and Hg, and up to 150 µg/ml Cu and Zn. Cultures of selected PGPR strains were observed to produce pyruvic acid, acetic acid, gluconic acid, lactic acid and citric acid when analysed by HPLC. PGPR isolates in pot trial experiments increased plant dry biomass up to 5-27%, root length 14-154% and shoot length up to 9-70% against control plants.

Introduction

The multiplying population is exerting immense pressure on agricultural lands for higher crop yields, which results in ever increasing use of chemical fertilizers. These agents are costly and create environmental problems. Consequently, there has recently arisen a renewed interest in environmental friendly agricultural practices (Karlidag *et al*., 2007). Long-term studies show that intensive application of chemical fertilizers lead to reduction in crop production. This reduction is mainly due to increasing soil acidity, decreasing biological activities and changing the soil physical characteristics and diminished microelements (Adediran *et al*., 2004). Microorganisms have a vital role in agriculture as they promote the exchange of plant nutrients and reduce application of chemical fertilizers as much as possible. Beneficial plant-microbe interactions in the rhizosphere can influence soil fertility (Dastager *et al*., 2011). Application of such

microbes as a biofertilizer may contribute to minimize the use of expensive fertilizers which can lead to create change in soil physiology (Serpil, 2012). Plant growth promoting rhizobacteria (PGPR) of many plant species and confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases caused by plant pathogenic fungi, bacteria, viruses and nematodes (Kloepper *et al*., 2004). In the rhizosphere, only 1-2% of bacteria promote plant growth (Antoun and Kloepper, 2001). Plant growth promoting bacteria are the natural potential resource which colonize roots of plants and stimulate growth and yield directly and indirectly (Afzal and Bano, 2008) Indirect plant growth promotion is mediated by antibiotics or siderophores produced by PGPR that decrease or prevent the deleterious effects of plant pathogenic microorganisms (Leong, 1986; Sivan and Chet, 1992). Whereas, Direct promotion of growth by PGPR including production of metabolites that enhance plant growth such as auxins (Ali *et al*., 2010), cytokinins, gibberellins and through the solubilization of minerals (El-Hadad *et al*., 2010). Phosphate solubilizing bacteria have been shown to enhance the solubilization of insoluble P compounds through the release of organic acids and phosphatases (Sharma, 2005). Organic acid production is the main mechanism by which phosphate solubilizing bacteria mobilizes P from sparingly soluble phosphate (Khan *et al*., 2007).

A considerable number of bacterial species from the rhizosphere have been isolated and their efficiency for improving plant growth has been assessed (Kloepper *et al*., 1989; Amor *et al*., 2008). However, fewer reports have been published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess, and tolerance to heavy metals (Zhuang *et al*., 2007; Glick, 2003). There is a profound need to isolate endogenous strains which can tolerate environmental stresses like salinity, temperature, pH, and heavy metals.

Promotion of plant growth by bacteria is well documented (Reed and Glick, 2004; Babalola *et al*., 2007; Babalola, 2010), and more recently PGPR have been successfully used to reduce plant stress in metal contaminated soils. Some microbial communities have the ability to sequester heavy metals and therefore may also be useful in bioremediating contaminated soils (Hallberg, and Johnson, 2005; Umrania, 2006). When microbes are used to bioremediate a contaminated site, plant-associated bacteria can be potentially used to improve phytoextraction activities by altering the solubility, availability, and transport of heavy metals, and nutrients as well, by reducing soil pH and releasing chelators (Ma *et al*. 2011).

Several studies have reported that the influence of PGPR is sometimes crop or niche specific or their benefits are limited due to the climatic variability and inconsistency of soils (Khalid *et al*., 2004; Wu *et al*., 2005). Therefore, in the present investigation attempts were directed towards selection of microbes exhibiting the highest number of traits associated with the plant growth promoting ability under *in vitro* conditions.

Materials and Methods

Isolation

Plants growing in rocky terrains, agricultural farms and on concrete structures were uprooted gently and soil attached to the roots was removed followed by preparation of serial dilutions. Dilutions were spreaded on nutrient agar and the plates were incubated at 30°C for 48 h. A total of 30 different colonies were isolated on nutrient

agar (NA) and isolated in pure by repeated culturing and maintained in 20% glycerol at -4°C temperature. Potential isolates were screened and selected on the basis of halo zone produced in Pikovskaya agar (Pikovskaya, 1948). Strains were assessed for colony morphology, Gram reaction and biochemical characterization.

Inoculum preparation

Bacterial isolates were grown in tubes containing 3 ml Nutrient broth (NB) and incubated overnight at 30ºC on shaker (120 rpm). Cultures were centrifuged at 10,000 rpm for 10 min and washed with 1 ml sterile 0.85% NaCl to remove free Pi present in medium. The bacterial pellet was re-suspended in 1 ml of 0.85% NaCl and was used as inoculum in the experiments.

Qualitative Phosphate solubilization

Primary phosphate solubilizing activity of the isolates was evaluated by growing microorganisms on Pikovskaya's agar for 72 hours at 30°C. Bacterial isolates that produced organic acids were identified by their ability to produce change in color of methyl red pH indicator (added at a concentration of 0.03%), from yellow (pH 8.0) to red (pH 5 or below) on Pikovskaya agar plates.

Qualitative Phosphate solubilization

Quantitative estimation of P solubilization was carried out, by inoculating 1 ml of bacterial suspension (3×10^7 cfu/ml) in 50 ml of National Botanical Research Institute Phosphate (NBRIP) broth (Nautiyal, 1999), in 150 ml Erlenmeyer flasks, and incubated for 7 days on shaker (120 rpm) at 30°C. Cultures were then centrifuged at 10,000 rpm for 10 minutes, and the Pi content in the supernatant was spectrophotometrically estimated by Vanado-molybdate method (Gulati *et al*., 2008), and pH of the medium was determined.

Indole acetic acid Production

Indole acetic acid produced by bacteria was determined as described by Brick et al. (Brick *et al*., 1991). Bacterial cultures were grown in NB amended with tryptophan (100 µg/ml) at 30°C for 48 h on shaker (120 rpm). The cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2 ml) was mixed with two drops of *o*-phosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink color indicated IAA production and A_{530} was recorded.

Production of ammonia

Bacterial isolates were grown in peptone water. 1% inoculum was added to 5 ml of peptone water in each tube and incubated for 72 h at 30°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Production of HCN

Isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (Lorck, 1948). Briefly, NA was amended with glycine (4.4 g/l) and bacteria were streaked on modified agar plates. Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid was placed at the inner surface of the lid of the Petri plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of orange to red color indicated HCN production.

Production of catalase

Bacterial cultures were grown in a NA medium for 24 h at 30°C. The cultures were mixed with 3-4 drops of H_2O_2 in test tubes to observe the effervescence.

Chitinase production

Chitin powder (Himedia Labs Ltd.) was added slowly to 10 M HCl and kept overnight at 4°C with vigorous stirring. The suspension was added to chilled ethanol with vigorous stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 10000 rpm for 20 minutes and washed with sterile distilled water until colloidal chitin became neutral (pH 7.0). It was dried to powder and stored at 4°C until further use (Berger and Reynolds, 1958).
Chitinase production was determined in NA containing colloidal chitin. Bacterial cultures were streaked on solidified agar plates and incubated for 7 days at 30°C. The ability of chitinase production was shown by a clear halo around bacterial colonies (Roberts and Selitrennikoff, 1988).

Amylase production

Amylase production was evaluated on starch agar plates containing in g/l peptone 5, beef extract 3, soluble starch 2 and agar 15 (Capuccino and Sherman, 2001). PGPR isolates were streaked on starch agar plates and incubated for 48 hours at 30°C. Amylase production was detected by flooding the plates with iodine solution (Hols *et al*., 1994).

Lipase production

The cultures were streaked on tributyrene agar (1% tributyrene, 0.5% peptone, 0.3% beef extract and 2% w/v agar) and incubated at 30°C for 72 hours. Development of clear zones around the microbial colonies indicated lipase activity.

Cellulase production

PGPR strains were grown on CMC (Carboxyl methyl cellulose) agar containing (g/l) KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NH_4NO_3 0.3, CMC 10 and Agar 15. The pH was adjusted to 7 with 1 M NaOH. The streaked CMC agar plates were incubated at 30°C for 5 days. At the end of incubation, agar medium was flooded with an aqueous solution of Congo red (1% w/v). Formation of clear zone indicated cellulose degradation.

Heavy metal tolerance

The four selected bacterial strains were tested for their resistance to heavy metals by agar dilution method (Cervantes *et al*., 1986). Freshly prepared NA amended with soluble heavy metal salts namely Hg, Co, Cu, Pb, Zn and Cr (50 to 400 µg/ml) and spot inoculated with overnight grown cultures and incubated at 30ºC for 48 h.

Organic acid analysis

50 ml NB in 250 ml Erlenmeyer flask was inoculated (1%) and incubated at 30°C for 72 h on shaker (120 rpm). Culture supernatants were filtered through 0.2 µm nylon filter and 20 µl samples were injected in the HPLC instrument (Shimadzu) equipped with C_{18} reverse phase silica column. Mobile phase consisted of 5 mM H_2SO_4 and the flow rate was 1 ml/min and oven temperature was maintained at 40°C.

Organic acids were detected by monitoring at A_{210}. Solutions of pure organic acids (gluconic, acetic, pyruvic, lactic, citric and succinic) were separately injected and their peak retention times were compared with that of the samples.

Antifungal activity

Bacterial culture was potted over the surface of potato dextrose agar (PDA) plates and after 2 days of incubation at 30ºC, the plates were inoculated with PDA plugs cut from 48 h fungal cultures. Control plates were inoculated only by fungi. Plates were incubated at 25ºC and examined for evidence of fungal growth inhibition. Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria.

Pot experiment

A bioassay-based determination of the plant growth promotion ability of the isolates was conducted using mung bean seedlings in sterile soil under glasshouse conditions. The mung bean seeds were surface sterilized in 70% ethanol for 2 minutes and in 0.2% $HgCl_2$ for 5 minutes followed by ten times washing with sterile tap water. Pure cultures were grown in NB at 28°C and diluted to a final concentration of 10^8 cfu/ml with sterile saline solution. The surface sterilized seeds were coated by immersing in the appropriate PGPR suspension (10^8 cfu/ml) for 45 min on a rotary shaker (120 rpm), air-dried, and sown immediately. Control seeds were treated with sterile distilled water. Seeds were sown in plastic pots (15 cm diameter) containing 6 kg of sterile soil (pH 7.2, organic carbon 2.6%, available P 51.2 kg/ha, available K 197.57 kg/ha, iron 34.44 mg/kg) and placed in a glass house. Thinning of seedlings was done 7 days after sowing and ten seedlings per pot were maintained throughout the experimental period. The soil was moistened to 50% of its water-holding capacity. The whole experiment was conducted in three independent trials. For each treatment, the plants of each pot were harvested 3 weeks after the emergence of seedlings and washed; morphological characteristics of each plant were recorded: plant height, root length, dry shoot and root weights. At harvest, the root system was separated from shoots, and both were oven-dried overnight at 65°C and dry weights were recorded against the control.

Bacterial identification

Identification of the isolates was determined on the basis of 16S rDNA sequence analysis. The DNA of the isolates was extracted according to the procedure described by Sambrook et al (Sambrook and Russell, 2001). Amplification of the gene was carried out by PCR using 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') universal primers according to Turner et al (Turner *et al*., 1999), using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. Consensus sequence of approximately 1300 bp 16S rDNA gene were generated from forward and reverse sequence data using aligner software. Alignment of the 16 rDNA sequence was conducted by using the BLASTN program from NCBI web site (http://www.ncbi.nlm.nih.gov). Based on maximum identity score the sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 (Tamura *et al*., 2007).

Statistical Analysis

All the experiment were performed in triplicate and repeated at least once. Appropriate controls were also included. The data collected were statistically analyzed using a completely randomized design in the case of the pot trials. The means were compared using a least significant difference test. The correlation

coefficients between a pair of trait were determined and the significance of the correlation determined using ANOVA. All the statistical tests were performed at $p < 0.05$, using the software INSTAT.

Results and Discussion

Rhizospheric soil along with roots samples from plants growing at different sites situated near Rajkot district of Gujarat, were collected and used for the isolation of PGPR having multiple plant growth promoting activities. The attempts yielded 30 isolates, exhibiting plant growth promoting activities. The isolates were identified based on biochemical characteristics (Table 1) according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). All the isolates were tested for their PGP traits like solubilization of insoluble inorganic phosphate, produce plant growth promoting substances. Four most prominent isolates (T-2, T-4, BR-1 and RR-1) were identified on the basis of their 16S rDNA gene sequence.

Molecular identification of PGP Isolates

After DNA sequencing, the 16S rRNA gene sequence comparisons with the available sequences were performed using Basic Local Alignment Search Tool (BLAST) network services of NCBI, USA database which resulted in the maximum score identities ranging from 2423-2573 with 99 to 100% identities. The direct sequencing of amplified PCR products resulted in molecular identification of growth promoting bacteria as *Bacillus subtilis* (RR-1), *Pseudomonas sp. a-1-8* (T-2), *Klebsiella sp.* T-4 (T-4) and *Cronobacter malonaticus* BR-1 (BR-1).The sequences of 16S rRNA gene of the four isolates were submitted to NCBI, USA, database and Gene accession number were assigned to these strains (Table 2).

Plant growth promoting Characteristics of test isolates

Out of four different PGP isolates isolate T-4 and RR-1 showed positive PGP activities in relation to phosphate solubilization, indole acetic acid (IAA), fungal cell wall degrading enzymes, and antagonistic activity against *Fusarium sp.* (Table 3). T-2 did not produce chitinase and amylase and did not show antagonistic activity. Isolate BR-1 showed positive PGP traits except amylase and HCN production. None of the strains produced HCN. The four selected microbial strains T-2, T-4, BR-1 and RR-1 solubilized 363, 405, 355 and 378 µg/ml tri-calcium phosphate respectively after 5^{th} day of incubation in NBRIP medium. The pH of the culture medium decreased as a result of bacterial growth; lowering of pH coincided with increase in the amount of phosphate solubilizing activity. The pH was observed to decline from 6.8 to 3.0.

Strong correlation was observed between the decrease in pH of the medium and phosphate solubilizing activity (Fig 1). Phosphate solubilizing activity increased as the pH of the culture medium decreased. The pH of the medium after 2 days in the case of the isolate BR-1 and 4 days with the other three cultures stabilized around 3, The phosphate solubilization also became steady. The four isolates also produced IAA and at 48 h significant ($P < 0.05$) difference between the ability of isolates to produce IAA could be translated by the expression RR-1 > T-4 > T-2 > BR-1 (Table 4).

Heavy metal tolerance:

On the basis of multiple plant growth promoting activities, 4 bacterial isolates were evaluated for their heavy metal tolerance. The four isolates T-2, T-4, BR-1

and RR-1 tolerated 400, 200, 150 and 100 µg/ml of Zn respectively while for Pb, Cu, and Cr strains showed similar pattern of heavy metal tolerance. For T-2, tolerated 100 µg/ml Hg, whereas isolates T-4, BR-1 and RR-1 tolerated up to 50 µg/ml. The strains except strain RR-1, tolerated up to 50 µg/ml Co (Table 5).

Organic acid analysis

The culture supernatants of the selected PGPR strains revealed the production of mainly gluconic acid, pyruvic acid, citric acid and lactic acid (Fig. 2). Isolates exhibited the same pattern for the production of organic acids except RR-1, which did not produce lactic acid.

Pot experiments

Treated seedlings of mung bean were found to be comparably different from the untreated such as morphological parameters like plant height and root length as well as fresh and dry biomass. Results revealed that all the isolates significantly improved plant growth parameters. Significant increase in root length was observed with T-2 (14%), T-4 (68%), BR-1 (154%) and RR-1 (116%) as compared to control. Shoot length increased by 9% (T-2), 59% (T-4), 69% with BR-1 and 70% with RR-1. On the other hand, shoot fresh weight showed 4% increase with T-2, 89% with T-4, 119% and 105% with BR-1 and RR-1 respectively. Increase in root fresh weight with T-4 was 82%, BR-1 was 228%, and RR-1 showed 112% increase as compared to control plants but T-2 showed decreased root fresh weight of 14%. Test isolates also showed increase in root dry biomass ranging from 5-27% and in shoot dry biomass by T-2 was (10%), T-4 (67%), BR-1 (156%) and RR-1 (131%). BR-1 was the most effective in increasing plant growth with respect to the tested morphological plant traits. When these parameters were statistically analyzed by ANOVA results were found to be significant (Fig 3-5).

Worldwide, there is a profound need to explore varied agro-ecological niches for the presence of native beneficial microorganisms. Many studies have been undertaken to understand the nature and properties of these unique microbes harboring potential plant growth promoting traits. With increasing awareness about the chemical-fertilizers-based agricultural practices, it is important to search for region-specific microbial strains which can be used as a potential plant growth promoter to achieve desired product.

All the rhizobacterial strains isolated on the basis of their multiple plant growth promoting abilities from the plants growing in local environment because the actual composition of the microbial community in the root zone is dependent on root type, plant species, plant type as well as other selection pressures. Some of the above-tested isolates could exhibit more than eight or nine PGP traits, Reports are available that PGPR strains are able to express multiple beneficial functions (Kloepper and Schrot, 1978). The isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically (Joseph *et al*., 2007; Yasmin *et al*., 2007). Similar to our findings of multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored (Gupta *et al*., 1998).

The recognition of PGPR, a group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing plant yields has evolved over the

past several years to where today researchers are able to repeatedly use them successfully in the field experiments. It is experimentally proved that PGPR have positive effect on the growth of different crops and plants (Wu *et al*., 2008; Bhattacharyya and Jha, 2012).

It is well established fact that improved phosphorous nutrition influences overall plant growth and root development (Jones and Darrah, 1994). Hence, frequent application of soluble forms of inorganic phosphate is necessary for crop production and which leaches to the ground water and results in eutrophication of aquatic systems. In view of environmental concerns and current developments in sustainability, research efforts are concentrated on elaboration of techniques that involve the use of less expensive, though less bio-available sources of plant nutrients such as rock phosphate and by application of phosphate solubilizing bacteria and the agronomic effectiveness can be enhanced (Whitelaw, 2000).

Potential PGPR strains *Bacillus subtilis* (RR-1), *Pseudomonas sp.* a-1-8 (T-2), *Klebsiella sp.* T-4 (T-4) and *Cronobacter malonaticus* BR-1 (BR-1) isolated from the agricultural farms, rocky terrains and plants growing at concrete structure showed multiple PGP traits like phosphate solubilization, IAA production, ammonia and enzyme production. Furthermore the isolates showed organic acid production as it may help in mineralizing rock phosphate content and mineralized phosphate content in the range of 362-405 µg/ml under in *vitro conditions*.

Biosynthesis of IAA is not limited to higher plants. Organisms such as bacteria are able to make physiologically active IAA that may have pronounced effects on plant growth and development. The selected four strains showed production of IAA. About 80% of bacteria isolated from plant rhizospheres are able to produce indole-3-acetic acid. Like plants, L-tryptophan is also considered as the IAA precursor in bacteria, because its addition to IAA producing bacterial cultures promotes and increases IAA synthesis (Tsavkelova *et al*., 2007). Root exudates are natural source of L-tryptophan for rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere. Production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several PGPR belonging to the genera *Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Pantoea, Pseudomonas,* and *Serratia*. The root exudates of various plants contain rich supplies of tryptophan, which are used by the microorganisms for synthesis and release of auxins as secondary metabolites in the rhizosphere.

Another important trait of the microorganisms that influences plant growth is the production of ammonia and antifungal metabolites, which suppress fungal pathogens in the rhizosphere. In the current studies the bacterial strains also exhibited the antagonistic activity at 30°C and none of the isolates produced HCN.

Deposition into soil over a long period of time results in high concentration of metal in soil which adversely affects the microbial flora of soil (Matsuda *et al*., 2002). Heavy metal in general show blocking their functional groups or these metals modify the biological molecules in particular their active sites. But these metals when present in lower concentration are important for microorganisms as they supply required co-factors for metallo-proteins and enzymes (Nies, 1999). Microorganisms have developed the mechanisms to cope with a variety of toxic metals for their survival in the environment enriched with such metals.

Table.1 Biochemical characteristics of PGPR strains

Isolates	Grams reaction	Shape	Pigments	Lactose	Dextrose	Sucrose	Mannitol	H_2S Production	Indole	Methyl red	VP	Citrate	NO_3 reduction	Starch	Gelatin
T1	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
T2	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
T3	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
T4	-	S	+	+	+	+	+	-	-	+	-	+	+	+	+
T5	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
BR-1	-	R	+	+	+	+	+	-	-	-	+	+	+	+	-
TR-1	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-2	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-3	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-4	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-5	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-6	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-7	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-8	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
TR-9	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
RR-1	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
RR-2	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
VV-1	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
VV-2	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
VV-3	-	R	+	-	+	+	-	+	-	-	-	+	+	+	-
BNF-1	+	R	+		+	+	+	+	+	+	-	+	+	+	-
Al-1	-	R	-	-	-	-	-	-	-	+	-	+	-	+	+
Al-2	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
Al-3	-	R	-	-	-	-	-	-	-	+	-	+	-	+	+
Al-4	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
Al-5	-	R	-	-	-	-	-	-	-	+	-	+	-	+	+
Al-6	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
Al-7	+	C	+	-	-	+	+	-	-	-	+	+	-	+	+
Al-8	+	R	-	-	-	+	+	+	-	+	-	+	+	+	+
Al-9	+	C	+	-	-	+	+	-	-	-	+	+	-	+	+

(+) positive reaction, (-) Negative reaction

Table.2 Closest relatives (BLAST search) of PGPR strains T-2, T-4, BR-1, and RR-1

Isolate ID	Isolation source	16S rDNA fragment length (bp)	Genus/Species	Closest relatives	Similarity (%)	Gene bank accession No.
T-2	*Cajanus cajan*	1355	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* N141	99.5	KC109000
T-4	*Cajanus cajan*	1312	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* L17	99	KC109001
BR-1	*Ficus religiosa*	1396	*Cronobacter malonaticus*	*Cronobacter malonaticus* PM199	99	KC109002
RR-1	*Holoptelea integrifolia*	1320	*Bacillus subtilis*	*Bacillus subtilis* hswx89	99	KC109003

Table.3 Multiple plant growth promoting activities of PGPR strains isolated from the rhizospheric soil samples

Isolates	PSA	IAA	Amylase	Lipase	Cellulase	Catalase	Antagonis	Chitinase	HCN	Ammonia
T-2	+	+	-	+	+	+	-	-	-	+
T-4	+	+	+	+	+	+	+	+	-	+
BR-1	+	+	-	+	+	+	+	+	-	+
RR-1	+	+	+	+	+	+	+	+	-	+

(+) positive reaction; (-) negative reaction

Table.4 Production of IAA by the PGPR strains during their growth in modified nutrient broth

Isolates	IAA produced (µg/ml)	
	48 h	**72 h**
T-2	17±1.2[a]	16±0.98[a]
T-4	21±0.28[b]	21±1.90[b]
BR-1	14±1.07[a]	15±1.61[a]
RR-1	23±1.10[b]	20±2.04[b]

Table.5 Growth of PGPR strains on nutrient agar amended with heavy metals

Isolates	Heavy metals (µg/ml)					
	Zn	Pb	Cu	Hg	Cr	Co
T-2	400	400	150	100	400	50
T-4	200	400	150	50	400	50
BR-1	150	400	150	50	400	50
RR-1	100	400	150	50	400	----

Fig.1 Changes in the pH (Δ) and amount of phosphate solubilized (○) by the PGPR strains (A) T-2, (B) T-4, (C) BR-1 and (D) RR-1 during their growth in NBRIP medium. Values are means of three replicates of three independent experiments. Control was without culture inoculation

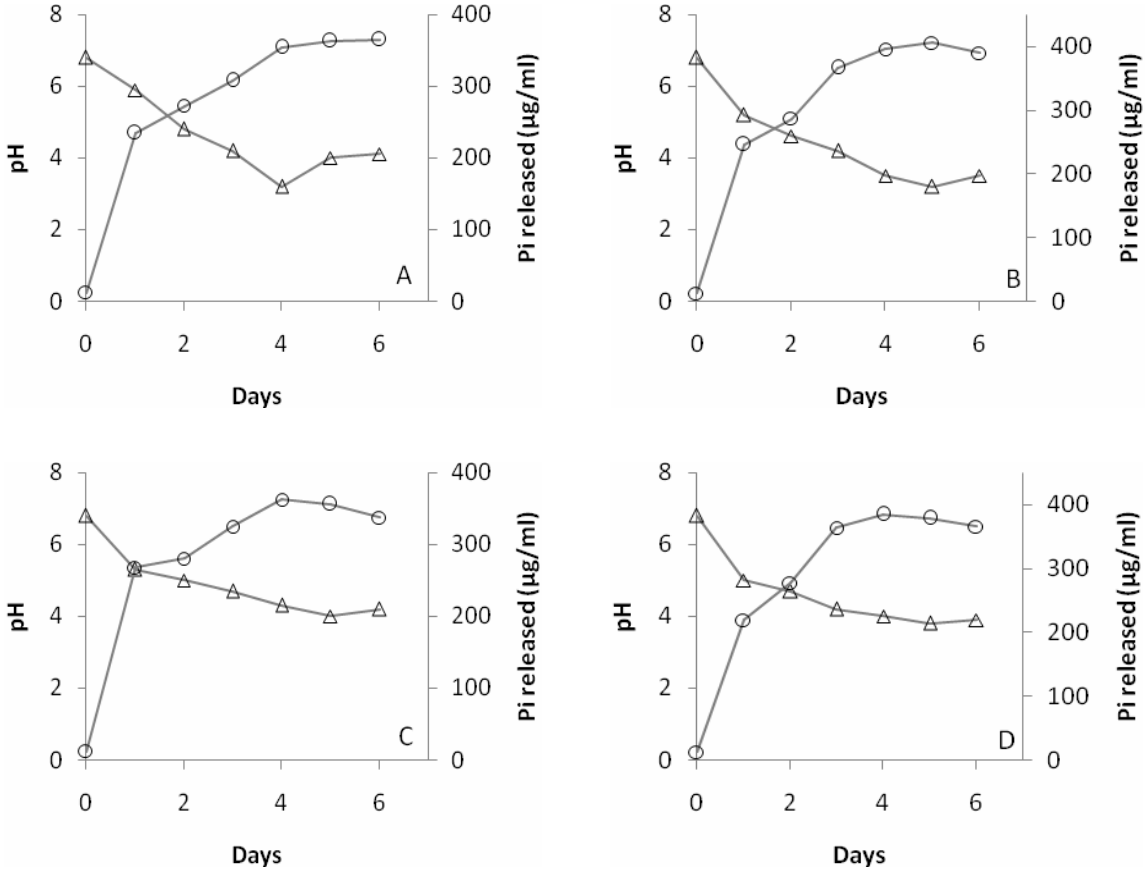
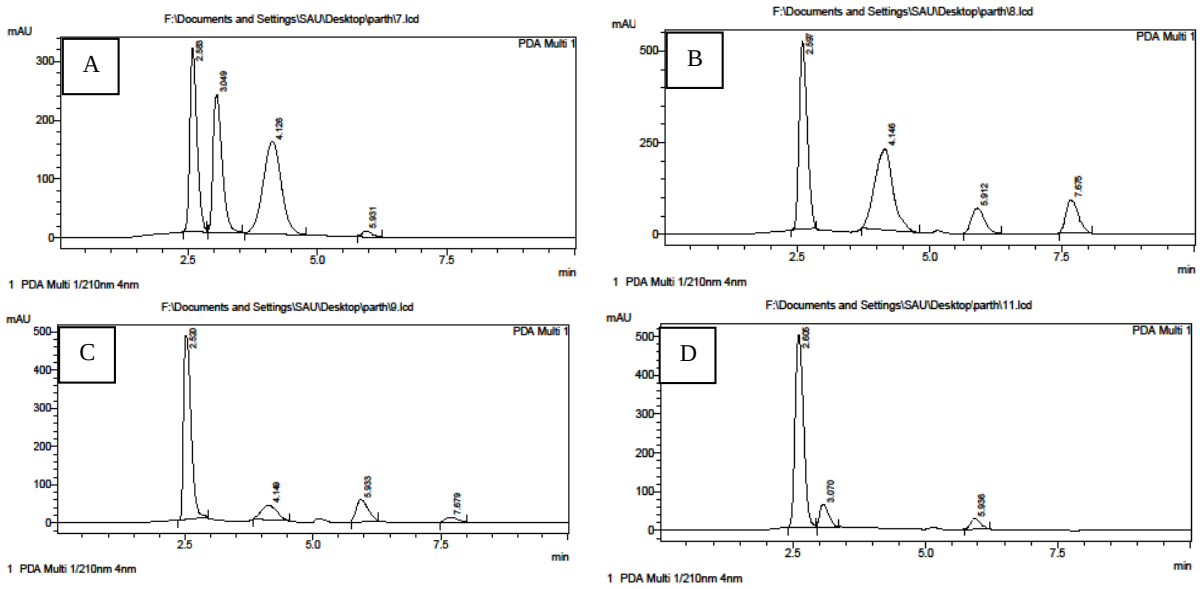


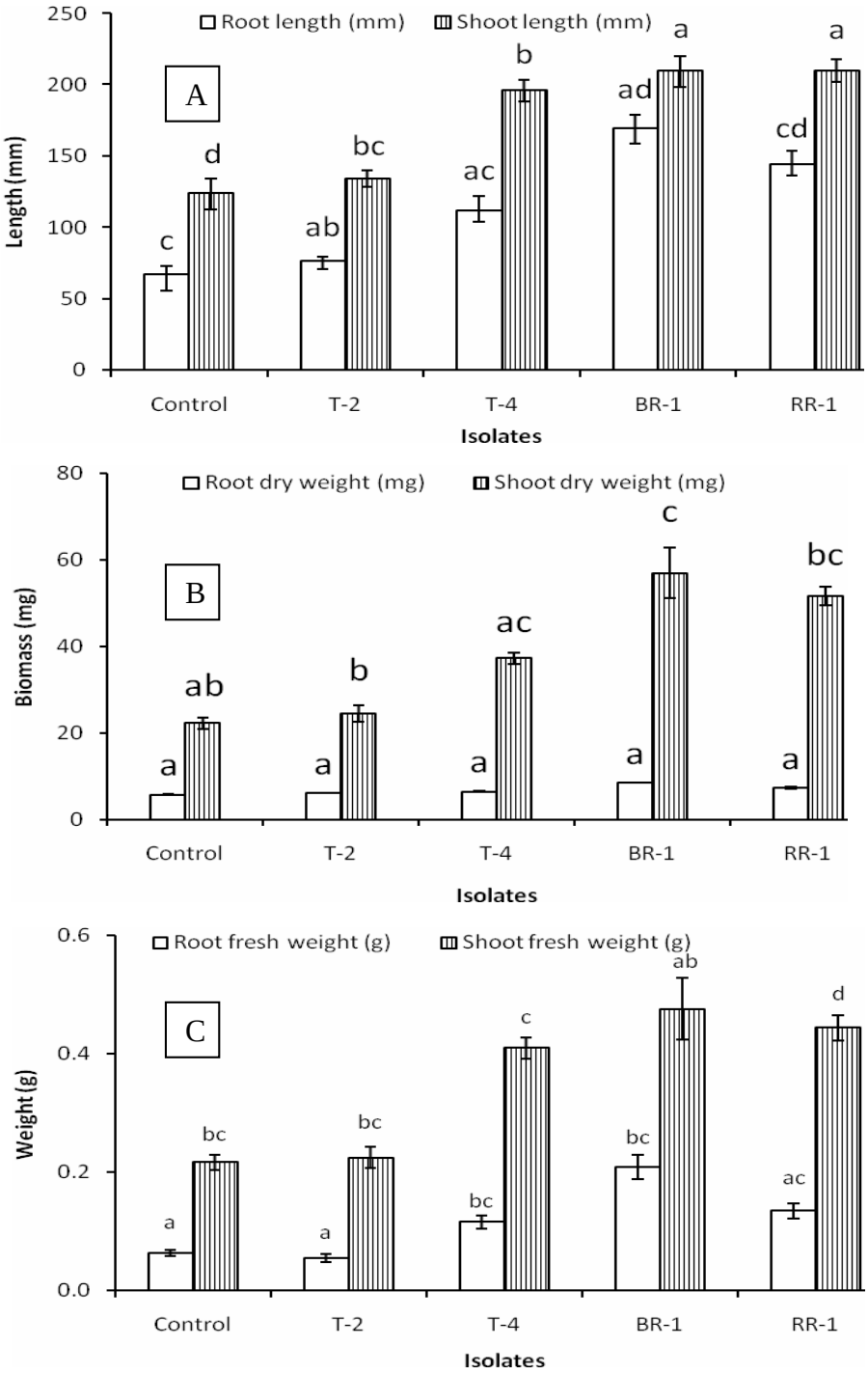
Fig.2 HPLC chromatograms of supernatants of 72 h grown cultures of PGPR strains (A) T-2 (B) T-4 (C) BR-1 (D) RR-1 growing in NBRIP medium



This study observed rhizobacteria tolerant to multiple heavy metals (Table 5), and exhibiting a couple of PGP activities (Table 3). Similar results have been reported in other crops such as potato, radish plants, sorghum and pearl millet by use of *Pseudomonas* strain (Burr *et al*., 1978; Raju *et al*., 1999; Niranjan *et al*., 2004).The metal-microbe interaction in natural environment is influenced by pH or organic matter content (Saeki *et al*., 2002). There are reports that have shown a high level tolerance to heavy metals by rhizobacteria (Raju *et al*., 1999). In the present study selected strains showed heavy metal tolerance up to 400 µg/ml. *Bacillus subtilis* (RR-1) tolerated Pb and Cr (400 µg/ml), for copper 150 µg/ml and tolerance exhibited towards Zn and Hg is 100 µg/ml and 50 µg/ml respectively. *Pseudomonas sp.* a-1-8 (T-2) is more heavy metal tolerant as compared to the other isolates, tolerating up to 400 µg/ml of Zn, Cr and Pb. All isolates tolerated Co up to 50 µg/ml. *Klebsiella sp.* T-4 and *Cronobacter malonaticus* BR-1 (BR-1) also exhibited heavy metal tolerance as they are Gram negative extracellular polysaccharide producing microbes. In other studies Gram negative bacteria are tolerant to zinc salts. For instance zinc tolerance by *Protobacteria, Actinobacteria* and *Bacteriodetes*. A varying level of resistance among the other PGPR (*Bacillus* and *Pseudomonas*) have also been reported (Niranjan *et al*., 2004).

Results of the pot trial study further justified the use of selected four strains and they significantly ($p<0.05$) improved the mung bean growth and development under glass house conditions. PGPR isolates increased plant dry biomass ranging from 5-27%, root length 14-154% and shoot length 9-70% against control plants. From the PGPR isolates *Cronobacter malonaticus* BR-1 improved plant growth by increasing root length, shoot length and dry biomass as compared with control and other isolates, may be due to the production of EPS in higher amount. Other studies suggest that the production of EPS possibly enhance water retention in the microbial environment and seems to regulate the diffusion of carbon source such as glucose (Saeki *et al*., 2002).

Fig.3 Root and Shoot (A) elongation (B) dry weight (C) fresh weight of mung beans cultivated from control seeds (uncoated) and seeds coated with PGPR strains. Results are expressed as means ± SD (n=30). One way ANOVA was performed for each plant section with different letters are significantly different from each other ($P < 0.05$)



PGPR inoculated mung bean plants have shown to improve shoot length, fresh weight, number of pods and seed weight (Wani *et al*., 2010). It has also been reported that inoculation of *V. radiata* with rhizospheric isolates significantly enhanced the plant growth under natural environmental conditions in pot trials (Yilmaz, 2003; Amellal *et al*., 1998). The improved plant growth due to PGPR inoculation on different crop plants has also been reported by several workers. Faisal and Hasnain (Ali *et al*., 2009) demonstrated that *B. cereus* inoculations significantly enhanced growth and yield of *V. radiata* under *in vitro* and wire house conditions. In another study, PGPR strains when co-inoculated with *Rhizobium* increases the vegetative growth and grain yield of *V. radiata* (Shahroona *et al*., 2006).

This study illustrates the significance of rhizobacteria under *in vitro* conditions for multiple PGPR traits and their evaluation under controlled conditions in a pot trial experiment. This led to the selection of effective PGPR isolates *Bacillus subtilis* (RR-1), *Pseudomonas sp.* a-1-8 (T-2), *Klebsiella sp.* T-4 (T-4) and *Cronobacter malonaticus* BR-1 (BR-1). Their multiple PGPR traits could prove effective in improving the plant growth parameters. Isolates may improve fertility of heavy metals contaminated sites as they showed tolerance against heavy metals. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers and these PGPR isolates may be used as biofertilizers to enhance the growth and productivity of for commercially grown plants under local agro-climatic conditions.

In addition to these traits, plant growth promoting rhizobacterial strains must be rhizospheric component, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. The results obtained *in vitro* cannot always be dependably reproduced under field conditions. It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promote root, shoot growth and yield. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits under field condition is in progress.

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