RESEARCH ARTICLE

Evaluation of indole acetic acid and phosphate solubilisation by plant growth promoting Rhizobacteria (PGPR): Potential use as Biofertilizer

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers. A total of six plant growth promoting rhizobacteria (PGPR), designated as PGPR1, PGPR2, PGPR3, PGPR4, PGPR5, PGPR6 were isolated from the rhizosphere soil and were screened for production of indole acetic acid (IAA) and inorganic phosphate solubilisation. The isolate (PGPR6) showing both the activities was selected and characterised. The isolate was identified as *Pseudomonas fluorescence*. The amount of IAA produced and phosphate solubilised by PGPR6was estimated by standard methods. PGPR6produced 20mcg/ml of IAA and released 147mcg/ml of solubilised phosphate. The effect of varying concentrations of L-tryptophan and tricalcium phosphate on plant growth promoting activities of PGPR6 was studied on mung (*Vignaradiata*) seedlings. The optimum amount of L-tryptophan required by the isolate was found to be 5mg/ml. It was found that 0.5% is the optimum concentration of tricalcium phosphate required by the organism to support root development. The shoot length, root length, wet and dry weight of the seedlings were measured and recorded. The data was represented in graphical form. Pot experiment was conducted to evaluate the effect of PGPR6on vigour index of plant. The vigour index of 'TEST' seed inoculated with PGPR6 showed higher value (1654) as compared to 'CONTROL' (521.05). Hence, *Pseudomonas fluorescence* is a potential PGPR which can be used as a biofertiliser.

Keywords : IAA, phosphate solubilisation, PGPR, biofertiliser

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonise plant roots and benefit plants by providing growth promotion, directly or indirectly, The common traits include production of plant growth regulators (auxin, gibberellin, ethylene etc.), siderophores, HCN, antibiotics and inorganic phosphate solubilisation. Inoculation of crop plants with certain strains of PGPR at an early stage of development improves biomass production through direct effects on root and shoots growth. There has been an increasing number of PGPR being commercialized for various crops.

Pseudomonas fluorescence is ubiquitous bacteria in agricultural soils and has many traits that make them well suited as PGPR. They are gram negative, motile, rod shaped bacteria and have diverse phytobeneficial traits. Their plant growth promoting activities include production of HCN, siderophores, protease, antimicrobials, plant growth promoting hormones such as IAA, phosphate solubilizing enzyme. Hence, they plays an effective role in stimulating yield and growth traits of crops and provides significant increases in fresh and dry masses (Rekha *et al*., 2010).

IAA is one of the most physiologically active auxins, produced by L- tryptophan metabolism by several microorganisms including PGPR (Sadaf *et al*., 2009). Effects of plant growth regulators including IAA on the plant will be concentration dependent. Farah etal., 2004 screened local isolates of *Pseudomonas sp.* for their intrinsic ability to produce IAA in the presence of varying amounts of L-tryptophan and their effect on root elongation of germinating seeds of test plants. Solubilisation of inorganic phosphate is another major phytobeneficial trait of *Pseudomonas fluorescence.* It is generally accepted that the mechanism of mineral phosphate solubilization by phosphate solubilising bacterial strains is associated with the release of low molecular weight organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Chen *et al*., 2005). PGPR can improve the nutrient use efficiency of fertilizers and allow reduced application rates of chemical fertilizers. The use of PGPR isolates as inoculants biofertilizers is beneficial for crop cultivation as they enhance growth of crop and by inducing other plant growth promoting traits. Applying PGPR as biofertilizer affects beneficially the yield and growth of crop plants in field conditions (Yildirim *et al*., 2009, Subramaniam Gopala-krishnan *et al*. 2015). Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPR is advantageous in the sustainable agricultural practices.

MATERIALS AND METHODS

Isolation of PGPR

Soil sample was collected from the rhizosphere of Country borage (*Coleus aromaticus*). The isolation of PGPR was done on Luria Bertani agar and King's B agar according to the method described by (Ashrafuzzman *et al*., 2009). The plates were incubated at 28±2°C for 48 hours. Shape, size, elevation, surface, margin, color and pigmentation etc. were recorded. Well isolated bacterial isolates were streaked onto fresh slants for maintenance and further characterization. The King's B agar plates were exposed to long wavelength UV light (365nm) for few seconds and the colonies exhibiting the fluorescence were picked up and streaked on to fresh King's B slants.

Screening for IAA production

Pure colonies of each isolate were grown in 100ml flasks containing 25ml of specific medium with and without L- tryptophan (5mg/ml). The screening for IAA was done according to method described by (Janardhan *et al*., 2010). Development of pink colour indicates IAA. The light absorbance was measured spectroscopically at 530nm.Amount of IAA produced was calculated

using the standard curve prepared with known concentration of IAA (Suresh *et al*., 2010).

Screening for phosphate solubilisation

Solubilisation of phosphate was determined by spot inoculation ofeach bacterial isolate on Pikovskayaagar medium (Pikovaskaya, 1948) (one isolate per plate) and incubated at 28 ± 2°C for 5 days and measuring the clear/halo zone around the isolate. Quantitative estimation of phosphate was carried out following ammonium molybdate-ascorbic acid method as described (Knudsen and Beegle, 1988).

Identification of PGPR isolate

Isolate showing positive for both IAA and phosphate solubilisation was identified by morphological and biochemical characterisation as described in Bergey's Manual of Determinative Bacteriology.

Seed germination test

Mung bean seeds were washed thoroughly under tap water and surface sterilized (Karnwal, 2009). The seeds were soaked in 18 hour old bacterial culture adjusted to 0.1 OD at 530nm (10^6cfu/ml) for 24 hours. The seeds were then transferred to water agar for germination at (28±2°C) for 24hours.

Effect of varying concentrations of L-tryptophan on seedling growth (Tryptophan optimization)

Jensson's seedling agar butts were prepared. Varying concentrations of L-tryptophan (1, 3, 5, 7, 9 mg/ml) were added to the butts aseptically (Farah *et al*., 2004). The bacteria coated seeds were transferred to butts aseptically (one seed per butt). Seed without bacterial coating was taken as control. The test tubes were cotton plugged and the lower portion of test tube upto the surface of butt was covered with aluminium foil to provide dark condition for seed germination. The agar butts were incubated for 5 days at room temperature (28±2°C) where sunlight is available. On the fifth day, seedlingswere removed without disturbing the root system and fresh weight of plant and dry weight of biomass were taken after drying samples to a constant weight in an oven (Fig3). The root length, shoot length were measured and recorded (Table1) (Ashrafuzzman *et al*., 2009). For studying the effect of varying concentrations of tricalcium phosphate on seedling growth, all the above procedures were kept same except that varying concentrations of tricalcium phosphate (0.3, 0.4, 0.5, 0.6, 0.7%) were used.

Pot experiment using PGPR isolate

Mung seeds were surface sterilized by method mentioned above. The seeds were soaked in 10 ml of bacterial suspension (10^6cfu/ ml) for 24 hrs and sterile blank nutrient broth served as control. Then the seeds were blot dried and sown in pots containing sterilized soil. The germination percentage was estimated at 10 days after sowing. Without disturbing the root system, the mung seedlings were depotted and observations on root length, shoot length, fresh weight of plant and dry weight of biomass were taken after drying samples to a constant weight in an oven and vigour index (VI) calculated (Dang, 2008).

VI = percent germination × mean total length of seedling (root length +shoot length)

RESULT

Isolation of PGPR

Six PGPR isolates were successfully isolated from the rhizosphere soil of Country borage (*Coleus aromaticus*). Five bacterial isolates were obtained from Luria Bertani agar medium. They were designated as PGPR1, PGPR2, PGPR3, PGPR4, PGPR5. One bacterial isolate was obtained on King's B agar medium. This isolate was designated as PGPR6 (Fig1).

Screening for IAA production

IAA production by PGPR isolates were analyzed. All six isolates showed positive for IAA production. PGPR6 was good producer of IAA whereas others showed poor IAA production. The

amount of IAA produced by PGPR6 was found to be 20mcg/ml.

Screening for phosphate solubilisation

Only PGPR6 showed the ability to solubilise phosphate. It produced a clear halo zone around the bacterial colony measuring about 3mm in diameter (Fig2). The amount of phosphate solubilised by PGPR6 was found to be 147mcg/ml.

Identification of PGPR isolate

PGPR6 showed positive for both IAA production and phosphate solubilisation. Hence it was selected for further characterisation. PGPR6 was identified as *Pseudomonas fluorescence.*

Effect of varying concentrations of tryptophan on seedling growth (Tryptophan optimization)

Highest seedling growth (15.3 cm) was observed at 5mg/ml tryptophan concentration followed by 7mg/ml tryptophan. However a decreased growth was shown at 9mg/ml tryptophan concentration, indicating that very high concentration of tryptophan may have a negative effect on seedling growth. Very poor growth was shown by seedlings at 1mg/ml tryptophan and in control tube. Hence 5mg/ml tryptophan concentration is optimum for plant growth. Highest wet weight (0.324g) and dry weight (0.024g) was observed at 5mg/ml L- tryptophan (Table1).

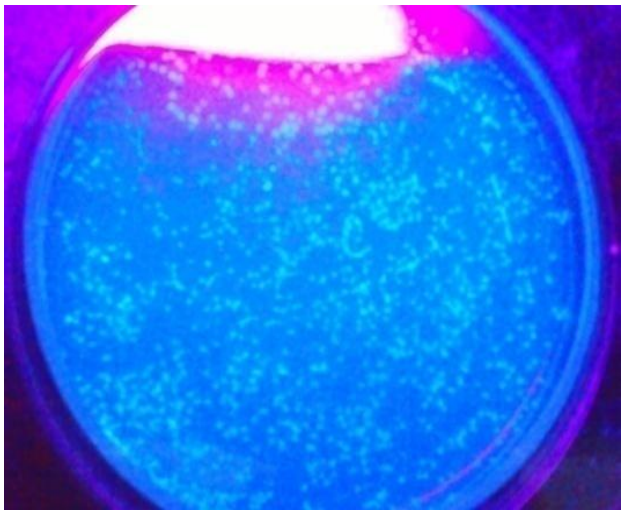

Fig 1:PGPR6 on King's B showing fluorescence under UV (365nm)

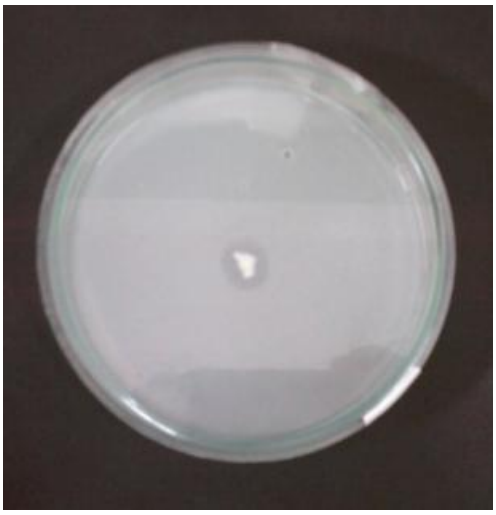

Fig 2: PGPR6 showing 3mm halo zone around colony

Fig3. Effect of different concentrations of L-tryptophan on seedling

Table 1: Effect of varying concentrations of L-tryptophan on seedlings treated with PGPR6

CONC (mg/ml)	LENGTH(cm)		WET WEIGHT(g)		DRY WEIGHT(g)	
	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT
CONTROL	3.5	1.5	0.036	0.014	0.002	0.001
1	5.5	3.2	0.045	0.022	0.004	0.001
3	10	3.0	0.283	0.031	0.018	0.002
5	**10.2**	**5.1**	**0.284**	**0.040**	**0.020**	**0.004**
7	9.8	4.9	0.275	0.038	0.016	0.004
9	6.5	2.8	0.150	0.027	0.007	0.002

Table2. Effect of varying concentrations of tricalcium phosphate on seedlings treated with PGPR6

CONC(%)	LENGTH (cm)		WET WEIGHT (g)		DRY WEIGHT (g)	
	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT
Control	10.9	3.0	0.280	0.035	0.020	0.005
0.3%	11.3	11.5	0.284	0.038	0.027	0.003
0.4%	10.6	6.3	0.283	0.092	0.026	0.005
0.5%	11.0	4.0	0.282	**0.107**	0.024	**0.006**
0.6%	11.0	3.1	0.280	0.095	0.019	0.006
0.7%	10.3	3.8	0.279	0.090	0.016	0.005

Fig4. Effect of different concentrations of tricalcium phosphate on seedlings

Fig5. Pot culture study

Effect of varying concentrations of tricalcium phosphate on the phosphate solubilising activity of PGPR6

As shown in Table2, the highest root wet weight (0.107g) and dry weight (0.006g) was observed at 0.5% tricalcium phosphate. Results reveal that all plants treated with PGPR6 showed increased shoot length, root length, dry weight, wet weight over control. Only at 0.7% concentration of tricalcium, seedling showed slight decrease in all parameters over control. Eventhough highest root length was observed at 0.3% tricalcium phosphate, the root and shoot dry weight and wet weight were considerably lower than 0.5%.

Hence 0.5% can be concluded as the optimum concentration of tricalcium phosphate required by the rhizobacteria to release highest amount of solubilised phosphate (Table2).

Pot experiment

The root length and shoot length of the mung bean plant was calculated after 10 days by taking the measurement of all the seedling growth and calculating its mean (Fig. 5). It was found that the test pot showed a shoot length of 9.92cm and root length of 6.62 cm. the germination percentage of the 'test' was found to be 100%. The plant growth promotion was assessed using Vigour index (VI).

VI = percent germination × mean total length of seedling (root length + shoot length).

Thus the vigour index of 'test' was found to be 165. In the control pot, the germination percent was found to be 85% and the mean shoot length was 3.26 cm and the mean root length was 2.87 cm. therefore by calculating the vigour index (VI) it was found to be 521.05. mung bean plant when treated with PGPR6 showed better growth characteristics as compared to 'control'. Thus it can be concluded that PGPR6 has the ability to promote plant growth. Field experimentation is required to conclusively prove the phytostimulatory potential of the isolate for its commercial exploitation as biofertiliser.

DISCUSSION

PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. (Sivakumar *et al*., 2007). Though the exact mechanism by which PGPR stimulate plant growth is not clearly established, although several hypotheses such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilisation and promotion of the mineral nutrient uptake are usually believed to be involved (Ashrafuzzman *et al*., 2009).

IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. IAA may function as important signal molecule in regulation of plant development (Ashrafuzzman *et al*., 2009). IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mirza *et al*., 2001). In present study, a total of 6 PGPR isolates were obtained. All PGPR strains showed positive result for IAA production. Among them, PGPR 6 was found to be good producer of IAA. Moreover isolates from rhizosphere are more efficient auxin producers than isolates from bulk soil (Sarwar *et al*., 1992). Most studies have shown that IAA biosynthesis is greatly influenced by L-TRP precursor. L-TRP is believed to be the primary precursor for formation of IAA in several microorganisms. Addition of L-TRP (an auxin precursor) to the media increased the auxin production by several fold (Farsana *et al*., 2009). IAA tryptophan monooxygenase catalyses theoxidative carboxylation of L-tryptophan to indole-3-acetamide which is hydrolyzed to indole-3-acetic acid and ammonia by indoleacetamide hydrolase via IAM pathway Hassan *et al*. (2009) stated that plants inoculated with the rhizobia together Ag+ ion and L-tryptophan gave the highest root dry weight and the uptake of N, P and K compared to non-inoculated control plants. The experiment indicated that rhizobia could be used as bioenhancer and biofertilizer for wheat production and usage of both Ag and L-tryptophan treatments together result in a significant increase on the uptake of N, P and K in comparison with using Ag+ ion and trpytophan alone and also in comparison with the blank. Patil, 2011 tested for the production of IAA in a medium with 0, 1, 2 and 5 mg/ml of tryptophan. A low amount of IAA production was recorded by *Azotobacter* strain without tryptophan addition. Production of IAA in *Azotobacter* increased with increase in tryptophan concentration from 1 to 5 mg/ml. In presence of 5 mg/ml of tryptophan, *Azotobacter* produced high levels of IAA. In the present study, highest seedling growth was

observed at 5mg/ml tryptophan concentration followed by 7mg/ml tryptophan. However a decreased growth was shown at 9mg/ml tryptophan concentration, indicating that very high concentration of tryptophan may have a negative effect on seedling growth. Very poor growth was shown by seedlings at 1mg/ml tryptophan and in control tube. The root development was highest at 5mg/ml tryptophan concentration. The amount of IAA produced by PGPR6 at 5mg/ml tryptophan was found to be 20mcg/ml.

Phosphorus is present in the form of insoluble phosphates and cannot be utilized by plants (Ashrafuzzman *et al*.,2009). Phosphate solubilisation ability of PGPR is considered to be one of the most important traits associated with plant phosphate nutrition. It is generally accepted that the mechanism of mineral phosphate solubilisation by phosphate solubilising bacterial strains is associated with release of low molecular weight organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Chen *et al*., 2005). Among the PGPR isolates only PGPR6 showed positive for phosphate solubilising activity indicated by formation of clear halo measuring 3mm around the colony. PGPR6 solubilised 147mcg/ml of inorganic phosphate at 0.5% Ca_3PO_4. Aftab *et al*. (2005) reported that mixture of *Pseudomonas* and *Bacillus* treatments resulted in statistically significant increase in seed phosphorus content over control. Grain yield and biological yield were significantly increased by the treatments and maximum yield was recorded when bacteria was used with phosphorus alone or along with organic matter. He concluded that phosphate solubilising microorganism alone or along with other combinations produced profound effect on grain and biological yield, tillers per m^2 and seed phosphorus content. The highest root wet weight and dry weight was observed at 0.5%Ca_3PO_4. Taken together the results suggest that *Pseudomonas fluorescence* (PGPR6) is a very efficient and promising PGPR for promoting plant growth and can be used as biofertiliser. However field experimentation is required to conclusively prove the phytostimulatory potential of the isolate for its commercial exploitation as biofertiliser

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