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PLANT GROWTH PROMOTING TRAITS AND PROSPECTS OF USING PHOSPHATE SOLUBILIZING *PSEUDOMONAS SP.* ISOLATED FROM LATERITE SOIL

Debasis Mitra[1], Arup Kumar Mondal[2], Manas Mondol[1] and Arijit Mukhopadhyay[1]*

[1]Department of Biotechnology, Oriental Institute of Science & Technology, Vidyasagar University, VIH campus, Rangamati, Paschim Medinipur, West Bengal- 721102 India
[2]Department of Biotechnology, Vidyasagar University, Rangamati, Paschim Medinipur, West Bengal- 721102 India.
*E-mail: aribak@rediffmail.com

ABSTRACT

We have isolated Pseudomonas sp. bacteria from Rangamati, Paschim Midnapur, West Bengal, India (La-22.27865^{0}N, Lo-87.37988^{0}E) which is predominantly lateritic in nature. It was screened for plant growth promoting (PGP) factors like phosphate solubilization, production of IAA, ammonia production and cell wall degrading enzyme activity (cellulose and protease) and growth inhibition against fungal and bacterial pathogens. The antibiotic test showed that isolate was susceptibility to amikacin, cotrimoxazole, chloramphenicol, tetracycline, vancomycin, ofloxacin, oxacillin, and gentamicin. It was found to be promising on all accounts. This work was carried out to explain the release of soluble phosphates and its effect on plant growth as a function of phosphate solubilization by fluorescent Pseudomonas sp. In the current study, It solubilized phosphate at a concentration of (175 µg/ml) in Pikovskaya's (PVK) broth after 4 days of culturing. The strain produced a fluorescent blue-green pigment. Effect of temperature (4-60^{0}C) and P^H (5-9) on this strain were also looked into. The biochemical characterization of the strain was done. Inoculation of chickpea and paddy rice seeds with the isolate significantly enhanced seed germination, plant root weight, plant height, and also fresh and dry weight in comparison with the control. The study suggested an uniqueness of this isolated strain and its potentiality as in developing a low cost eco-friendly multifunctional bio-fertilizer and bio-control agent in agriculture with high plant growth promoting activity.

Keywords: *Pseudomonas sp*., PGP, phosphate solubilization, *Oryza sativa* L.

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1. INTRODUCTION

Plant growth promoting (PGP) bacteria are very useful and beneficial for plant growth by a wide variety of mechanisms. Plant growth promoting activity are known to produce antibiotics, antifungal metabolites, enzymes, phenolics, signal compounds and other determinants of defense in response to pathogen attack (Niranjan et al. 2005; Maurhofer et al. 1998). Different plant-growth promoting rhizospheric bacteria, including associative and symbiotic bacteria such as *Azospirillum sp*., *Bacillus sp*., *Pseudomonas sp*., *Enterobacter sp.* groups have been used for their beneficial effects on plant growth (Kloepper et al. 1992; Ho¨flich et al. 1994). In the rhizosphere, bacterial population promotr plant health. They stimulate plant growth directly by nitrogen fixation, solubilization of nutrients, production of growth hormones, 1-amino-cyclopropane-1 carboxylate (ACC) deaminase (Kloepper et al. 1980). Several *Pseudomonas* species have been reported among the most efficient phosphate - solubilizing bacteria and as important bio - inoculants due to their multiple biofertilizing activities of improving soil nutrient status, secretion of plant growth regulators and suppression of soil borne pathogens (Verma et al. 2010; Trivedi et al. 2008). Independent of the mechanisms of growth promotion, PGPR colonize the rhizosphere, the rhizoplane (root surface), or the root itself (within radicular tissues) (Gray et al. 2005). It is well established that only 2-5% of bacteria promote plant growth in the rhizospheric zone (Antoun et al. 2001). Bacteria of diverse genera have been identified as PGP, of which *Bacillus sp*. and *Pseudomonas sp*. are predominant (Podile et al. 2006). PGP affect plant growth in two different ways, indirectly or directly. The direct promotion of plant growth by PGP entails either providing the plant with a compound that

is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Vessey, 2003; Glick, 1995). Biofertilizers with new concept of PGP have an important role in crop nourishment as well as enhancement of soil fertility by means of several mechanisms like biological nitrogen fixation, solubilization of native phosphorus, acquisition of essential macro and micronutrients with mineralization of organic manures/organic matter, production of plant growth promoting substances, disease control by suppression of soil borne phytopathogens and acceleration of other microbial activities in the soil (Elkoca et al., 2008; Verma et al., 2010). Chen et al. (2006) Note that phosphorus (P) is an essential macronutrients often limiting the plant growth due to its low solubility and fixation in the fertile land. Improving soil fertility by releasing bound phosphorus by microorganism's inoculants is an important aspect for increasing crop yield (Ahlawat, 2005; Ahmad et al. 2008). Phosphorus deficiency is a major constraint to crop production due to rapid binding of the applied phosphorus into fixed forms not available to the plants (Parani et al. 2012). Phosphorous release from insoluble phosphate reported for several soil microorganisms has been attributed mainly to the production of organic acids and their chelating capacity (Fiske et al. 1925; Goldstein et al. 1998). The aim of the present study was to explicate the release of soluble phosphates and its effect on plant growth as a function of phosphate solubilization by *Pseudomonas sp*. bacteria.

2. MATERIAL AND METHODS

The sample was collected from laterite soil of Rangamati, Vidyasagar University, Paschim Medinipur, West Bengal, India-721102(La-22.27865^{0}N, Lo-87.37988^{0}E). The soil sample was serially diluted and 100µl of dilution sample (10^{-6}) was plated onto nutrient agar media and pure culture was obtained by streaking four or five times in King's B medium.

The isolation strain was analyzed for its ability for phosphate solubilization, IAA (indole -3-acetic acid) production, ammonia production and HCN-SA production.

Phosphate solubilizing ability of the isolate (or1) was evaluated on Pikovskaya (PVK) medium incorporated with tri-calcium phosphate (TCP) [$Ca_3(PO_4)_5$] as insoluble phosphate. Quantitative phosphate solubilization was estimated Fiske et al. (1925) by Fiske and Subbarow method.

Indole acetic acid (IAA) production by bacteria was assayed calorimetrically using ferric chloride acid reagent in the presence of tryptophan (Dubey, 2007). Detection of ammonia production was done by adding 1.5 ml Nessler's reagent to a 48-h-old culture in (4%) peptone broth and recording the presence of the yellowish brown color (Meyer et al. 1992). Protease activity (casein degradation) was determined from clear zone in skim milk agar (skim milk powder-100.0g, peptone-5.0g, agar-15.0g, PH-7.2) (Dubey, 2007). Colonies were screened for cellulose activity by plotting on CMC (Carboxy Methyl Cellulose) agar and Czapek-mineral salt medium ($NaNO_3$-2.2g, K_2HPO_4-1.0g, $MgSO_4.7H_2O$-0.7g, KCl-0.5g, CMC-5.0g, peptone-2.5g, agar-18.0g, distilled water-1000ml, PH-6.0) (Cattelan et al. 1999; Aneja, 2003). HCN production was tested according Kremer et al. (2001) method described by Kremer and Souissi. *Pseudomonas sp*. was tested for salicylic acid, which plays an important role in signaling pathway leading to induced systemic resistance (ISR) (Meyer et al. 1992).

Plant growth promotion ability of the isolate applies on paddy rice and chickpea:

A local paddy rice (*Oryza sativa L*.) and chickpea (*Cicer arietinum L*.) seeds used for the experiments were obtained from Plant Tissue Culture lab, Dept. of Biotechnology, Oriental Institute of Science & Technology, Rangamati, Vidyasagar University, Paschim Medinapur, West Bengal, India-721102. *Oryza sativa L*., commonly name as Asian rice, is the plant species most commonly referred to in English as rice. One of the world's important staple crops and major part in the diet of more

than half the world's population, rice also has many medicinal uses (Rocheli et al. 2013). For another experiment Chickpea, *Cicer arietinum L.*, is an important pulse crop with synonym of bengal gram, garbanzo (Spanish), chana (Hindi) and chanaka (Sanskrit). Chickpea is the largest produced food legume in South Asia and the third largest produced food legume globally. India is the largest chickpea producing country accounting for 64% of the global chickpea production (Kloepper et al. 1980). It is grown in an about 30% of the national pulse acreage which contributes to about 38% of national pulse production in India. Chickpea plays a significant role in improving soil fertility by fixing the atmospheric nitrogen. It can fulfill 60-80% of its nitrogen requirement through symbiotic N_2–fixation to give high grain yield when grown in association with effective and competitive Rhizobium strain (Kyein-Boahen et al., 2002). Chickpeas are low in fat and most of this is polyunsaturated (Ahlawat, 2005).

Chickpea (*Cicer arietinum L.*) and paddy rice (*Oryza sativa L.*) seeds were used as plant materials. The seeds were surface sterilized in 70% ethanol for 30 second, in 2% sodium hypochlorite for 7 min and followed 8 times washing in sterile double distilled water.

The experiment soil sample was autoclave at 15 lb/inch^2 pressure for 15 mints and bacterized separately in sterilized 60ml culture tubes for 15 days. 200ml of bacteria inoculums, containing 3×108 cfu/ml, are separately centrifuged at 8000rpm for 15 mints (cooling centrifuged, REMI C-24 BL, India) supernatant are discarded. Bacterial pallets were washed four times with carboxy-methylcellulose (MERCK-MF8M581422) solution (1mg CMC in 100ml sterile distilled water) (Ramamoorthy et al. 2002). Then the bacterial solution is added separately with the sterilized soil sample kept in culture tubes (Abdul Baki et al. 1973). After 8 days incubation, PGP activities by selected microbes are assessed and results were calculated for this set. In another experiment (apply in Cicer arietinum L.) after 10 days result are noted.

Antibiotic profiling was done by disc diffusion method using – HIMEDIA (IC002-Icosa G-1 Plus). H_2S production by the isolate was detected on Kligler's agar by forming a black precipitate at the streaking or stabbing after using the P^H indicator phenol red in the medium (Aneja, 2003). To determine the effect of P^H (5-9) and temperature (4-60^{0}C), the extent of growth of the isolate was determined by measuring the $O.D._{660}$ of spent culture at the aforementioned conditions.

3. RESULTS AND DISCUSSION

The isolate was gram-negative, small rods and acid fast staining negative. By biochemical characterization, the isolate stain was identified as belonging to the genus *Pseudomonas*. Biochemical test in order to determine the physiology of the isolated strain, a series of biochemical tests were performed (Tab-1). To determine the range of P^H (5-9) and temperature (4-60^{0}C) that the bacteria can grow in Nutrient Broth (Aneja, 2003). The samples were incubated at 28^{0}C for four days. To determine the different enzymes that the bacteria may have, different tests were done. Urea broth was used to see if the bacteria can degrade urea. SIM medium were used to see if the isolates could degrade tryptophan to indole, using the enzyme tryptophanase, and to determine the production of H_2S and to see if the bacterium in motile. The Nutrient Gelatin Agar was used to see if the isolate produced the exo-enzyme gelatinase (Cardona-Cardona et al. 2010). To determine the carbohydrate utilization, assays were performed with sucrose, dextrose, maltose with phenol red as indicator. The ability to use glucose, lactose, and sucrose was measured using Triple Sugar Iron Agar (TSIA) slant, the production of H_2S and gas were also observed. The utilization of citrate as only carbon source was measured using Simmons Citrate Agar slants. To detect the conversion of pyruvic acid in acetone, the Methyl Red (MR) test was used and to see the fermentation of glucose and its transformation to pyruvic acid, we used Voges-Proskauer (VP) test. Phenylalanine deaminase production test

is positive, prepare phenylalanine agar plate and streak with culture and incubate at 37^0C for 48 hours. Add 4-5 drops of 10% $Fecl_3$, after 1 minute examine the colour. For $NO3^-$ reduction, prepare a nitrate broth; inoculate culture at 37^0C for 48 hours. Added nitrate test reagent, developed red colour (positive test result). These tests are described (Harley, 2007).

Table 1 Biochemical characterization of isolate

Sl. No.	Biochemical Test	Result
1.	MR-VP test	+
2.	Citrate test	+
3.	H_2S production	+
4.	Catalase test	+
5.	Oxidase test	+
6.	Starch hydrolysis	-
7.	Casein Hydrolysis	+
8.	Peroxidase test	+
9.	Indole test	+
10.	Urease test	+
11.	Phenylalanine deaminase	+
12.	Nitrate reduction	+

Legend: + Positive, - Negative

The antibiotic test of isolate strain to 12 antibiotics was determined on nutrient agar plates (peptone-0.5gm, beef extract powder-0.3gm, NaCl-0.3gm, agar-1.8gm, P^H-6.8) by disc diffusion method (Tab-2).

Table 2 Presence of susceptibility and resistant isolated strain

Sl. No.	Antibiotics	Susceptibility (in Diameter)	Result
1.	Tetracycline (TE-30µg)	24mm	S
2.	Vancomycin (VA-30µg)	10mm	S
3.	Cephalothin (CEP-30µg)	-	R
4.	Amikacin (AK-30µg)	29mn	S
5.	Cotrimoxazole (COT-25µg)	30mm	S
6.	Chloramphenicol (C-30µg)	20mm	S
7.	Teicoplanin (TEI-10µg)	-	R
8.	Penicillin (P-10µg)	-	R
9.	Ofloxacin (OF-5µg)	40mm	S
10.	Oxacillin (OX-1µg)	10mm	S
11.	Gentamicin (GEN-10µg)	30mm	S
12.	Linezolid (LZ-30µg)	-	R

Legend: S-susceptibility, R-resistant

Sugar fermentation is recommended for identification of isolated strain. The acid, alkali or gas production result a visible change in the inoculated isolated cultured (Tab-3).

Table 3 Sugar fermentation for acid and alkali/gas production

Bacterial Strain	Glucose	Sucrose	Lactose
Pseudomonas sp. Isolated	AG	±	±

Legend: A-acid only i.e. turned yellow, AG- acid + gas, - no change, ± variation reaction

The presence of ammonia is indicative of ammonification which is detected by the yellow to brown precipitate after adding Nessler's reagent (Figure-3, E).

After incubation (at 28^0C for 48 hours), adding 1ml of Kovac's reagent, after 2 minutes a red colour band appears at the junction of medium and reagent (Libbert et al. 1969). For confirm test, indole is diffused above the medium and paper strip turns pink if the strip is already impregnated with oxalic acid. The appearance of pink colour indicates the IAA production by the isolated strain. Cellulase production test (degradation of cellulose): a clear zone will be observed around the colony. Protease activity (casein degradation): a clear area surrounding the bacterial colony. Results are shown in the figure 3, F. Microbial production of HCN has been reported Ramette et al. (2003) as an important antifungal trait to control root infecting fungi. HCN production was recorded by our isolate *P. aerugonisa*, as evidenced by change in color of filter paper from yellow to reddish-brown after 2-4 days of incubation (Bakker et al. 1987).

Phosphorus is one of the vital nutrients for microorganisms next to nitrogen. Several species of bacteria (*pseudomonas sp.* and *Bacillus sp.*) degrade and solubilize the insoluble phosphate into soluble forms through the mechanism of secretion of organic acids e.g. acetic acid, glycolic acid, formic acid, lactic acid, succinic acid etc. (Rodriguez et al. 1999). These acids decrease pH and cause dissolution of bound form of phosphate (Sultana et al. 2004). The sources of phosphate in soil are both minerals (e.g. tricalcium phosphate, calcium phosphate, iron phosphate, hydroxy apatite and rock phosphate) and organic phosphates (e.g. phytin, calcium

glycerophosphate, phenyl phosphate, lecithin, etc.) (Dubey, 2007). In this study, isolated strain highly able to solubilize phosphate. It is solubilizing insoluble phosphate at the rate (Figure-1).

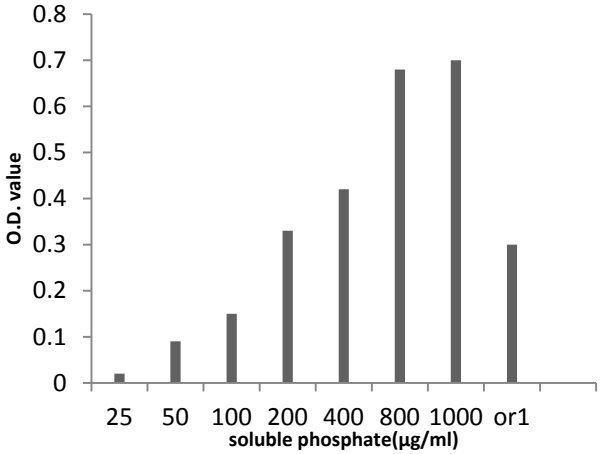

Fig. 1. Phosphate solubilization by or1

The overall performance of inoculated treatments revealed that seed treatment of paddy rice (*Oryza sativa L.*) and chickpea (*Cicer arietinum L.*) with inoculant isolated bacterium, as good phosphate solubilizers could give significantly better performance in respect of control (Tab-4&5). Use of the microorganisms to promote plant growth and to control plant pests continues to be an area of rapidly expanding research (Vessey, 2003). PGP mediated agriculture is now gaining worldwide importance and acceptance for an increasing number of crops and managed ecosystems as the safe method of pest control (Niranjan et al. 2005). (Figure-3, D & 3, G).

Table 4 Plant Growth Promoting treatments results in paddy rice (*Oryza sativa L.*) during eight days

Test tube no.	Seed: paddy rice (Oryza sativa L.)	Root Height (cm)	Shoot Height(cm)	Root weight(mg)
1.	OzS-Control	3.1	2.3	0.16
2.	OzS-I	4.2	5.3	0.22
3.	OzS-II	4.4	4.1	0.21
4.	OZS-III	1.7	4.6	0.23

Legend: OzS-control, I, II& III - apply Seed sample: paddy rice (*Oryza sativa L.*)

Table 5 Plant Growth Promoting treatments result in chickpea (*Cicer arietinum L.*) during ten days

Test tube no.	Seed: chickpea (Cicer arietinum L.)	Root Height (cm)	Shoot Height (cm)
1.	CrA-control	10± 0.5	24±0.8
2.	CrA-apply	14± 0.7	30±0.6

Legend: CrA- seed sample of chickpea (*Cicer arietinum L.*)

Catalase test was performed by taking a drop of 4% hydrogen peroxide was added to 48 hours old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick (Aneja, 2003). The effervescence indicated catalase activity. Isolated strain is able to degrade of sulphur containing amino acids (cysteine and methionine) for H_2S production. At 28^0C for 48 hours, appearance of black colour shows H_2S production. This black colour due to the production of H_2S from an ingredient of the medium (i.e. sodium thiosulfate) that then combines with another ingredient of the medium (ferrous ammonium sulfate),resulting in the formation of the black insoluble compound, ferrous sulfide (Dubey, 2007) (Figure-3, B).

When the isolate was subjected to temperature stress, it showed a zig-zag pattern. The optimal growth temperatures are 37^0C and 28^0C with practically no growth at 4^0C (Figure-2).

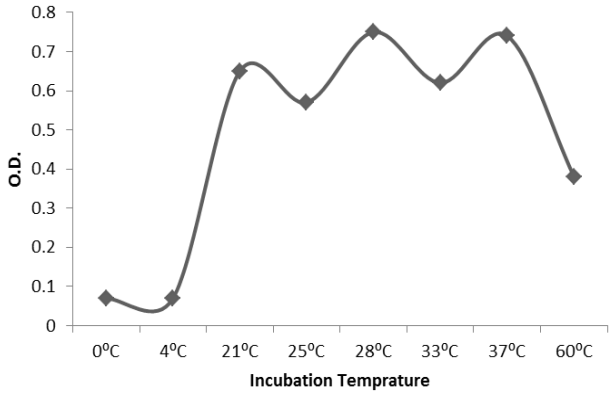

Fig. 2 *Pseudomonas sp*. Strain growth curve in temperature at 660 nm in UV/Visible Spectrophotometer

4. CONCLUSION

In our study, we used this bacterium as a bio fertilizer and phosphate solubuiliser. The biochemical characterization and

morphological study of the isolate shows that its Pseudomonas sp. bacteria. It is having a number of PGP traits. It is a good phosphate solubiliser. The blue green florescent pigment is prima facie anti-microbial agent (data not shown). It can be developed as an effective PGP agent and bio-fertilizer, though further investigations are needed.

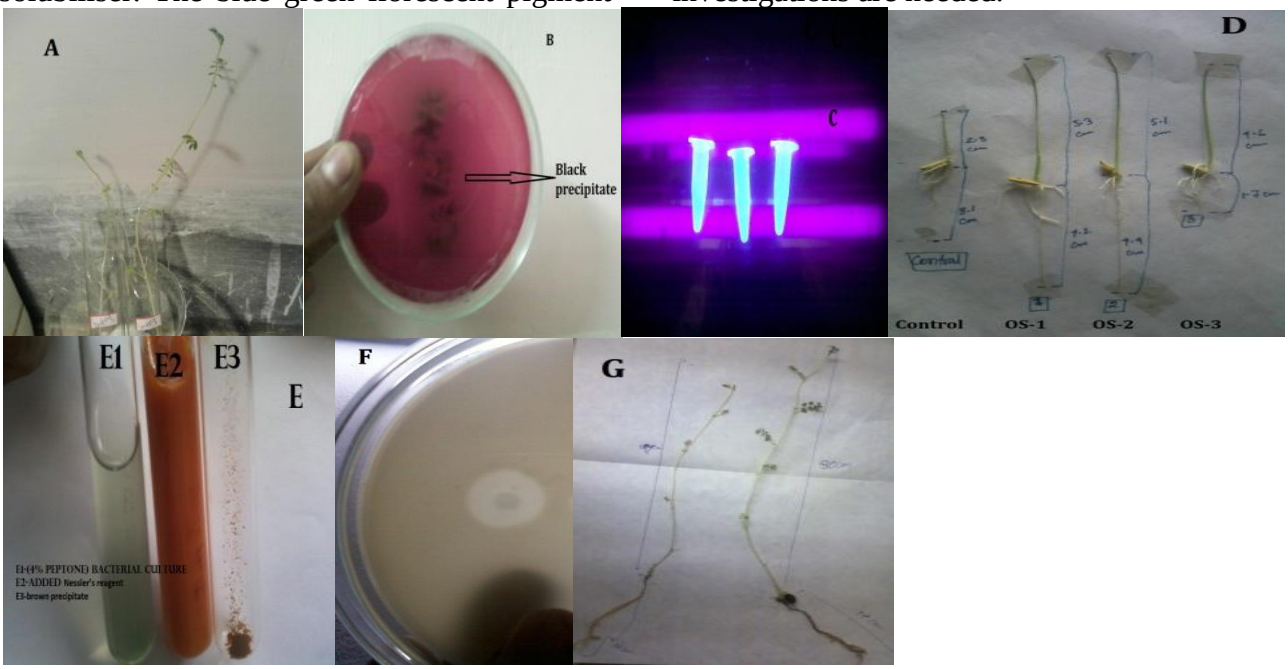

Fig. 3. A-applies (PGP) in chickpea (*Cicer arietinum L.*) result in 10 days, B-H_2S production, C-fluorescent in UV, D-Result of PGP applies: *Oryza sativa L.*, E-Ammonia production positive test, F-Clear zone in casein hydrolysis agar for Protease activity and G- Result of PGP in chickpea (*Cicer arietinum L.*)

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