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# EVALUATION OF PHOSPHATE SOLUBILIZING ABILITY OF THE BACTERIAL STRAINS ISOLATED FROM ROOT NODULES OF PIGEON PEA (*CAJANUS CAJAN*)

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## ABSTRACT

The *Bradyrhizobium* and endophyticstrains isolated from the root nodules of pigeon pea plants were tested *in-vitro* for phosphate solubilization activity on pikovskaya media. Out of total 15 strains, ten strains tested (S1, S3, S5, S6, S8, S9, S10, S11, S12 and S13) positive for phosphate solubilization ability by making halo zones around colony on medium after 96 hours. The strains S8, S9, S11 & S13 made larger halo zones in comparison to the other six strains *i.e.,* S1, S3, S5, S6, S10 and S12 which indicated that S8, S9, S11 & S13 had more ability to solubilize phosphate in comparison to other strains.Same experiments with these strains (S1, S3, S5, S6, S8, S9, S10, S11, S12 and S13) were performed in pikovskaya broth and optical density was measured at 475 nm wavelength after 24 to 96 days. The result showed the increase in OD value with increase in duration for all the strains. Highest OD was observed for the strain S11 (1.984) followed by S4 (1.824), S13 (1.759), S9 (1.747), S10 (1.554), S12 (1.421), S1 (1.401), S8 (1.332), S3 (1.366) & S6 (1.246) after 96 hours. It was also observed that there is perfect correlation between phosphate solubilization efficiency on solid and liquid medium *i.e.,* result of phosphate solubilization index obtained through pikovskaya agar plate was exactly similar to that obtained through liquid medium. This study showed that out of 15 bacterial strains tested for phosphate solubilizing ability, only nine *Bradyrhizobium* strains (S1, S3, S6, S8, S9, S10, S11, S12 and S13) and one endophytic strain (S5) showed their ability to solubilize the phosphorus.

## Introduction

Pigeon pea (*Cajanus cajan*) is a perennial legume of family fabaceae having a diploid genome (2n = 22) and center of origin in peninsular India (Vavilov, 1951). It is the preferred crop in dryland areas (Joshi *et al.* 2001) andan important leguminous crop of rainfed agriculture in all tropical and semitropical regions of the world. India is the main pigeon pea growing country which supplies nearly 90% of total world's production. India ranked first in area and production in the world with 79% (4.78 million ha) and 67% (3.6 million tons) of world area and production respectively (FAO statistics, 2014). Pigeon pea being a leguminous crop, have the ability to boost soil fertility through nitrogen fixation from its nodules and organic carbon in soil from the leaf fall and nutrients recycling (Snapp *et al.*, 2003). Plants always remain in close association with a number of soil microbes including bacteria & fungi which inhabit the rhizosphere as well as phyllosphere without causing any harm to the plants. Symbiotic nitrogen fixation is considered as one of the most important microbial activities in the soil because it provides nitrogen for the legume in the soil and contributes to the nitrogen cycle in the biosphere (Hardarson& Atkins, 2003). Pigeon peas are often described as non–specific in their *Rhizobium* requirements and can be effectively inoculated by the indigenous soil populations. However, inoculation of pigeon pea with *Bradyrhizobium* increases the number of primary branches, reduces the time to flowering and maturity and increases nodulation and grain yield. The presence of *Bradyrhizobium* with pigeon pea at the center of origin and in countries where pigeon pea was introduced, suggests that *Bradyrhizobium* is the original symbiont of pigeon pea.

A wide range of soil bacteria, which exerts beneficial effects on plants showing several plant growths promoting activities and leads to increased yields of a wide variety of crops are known as plant growth promoting rhizobacteria (PGPR) (Glick *et al.*, 1994). Plant growth promoting activities by PGPR include phosphate solubilization, zinc solubilization, potash solubilization, siderophore production, production of indole-3-acetic acid (IAA), nitrate reductase activity, by producing secondary metabolites such as antibiotics, hydrogen cyanide (HCN) and phytoalexins and as the biological control agent for phytopathogens (Arora *et al.*, 2001; Deshwal *et al.*, 2003). Plant growth promoting (PGP) rhizobacteria strains use these mechanisms alone or in combination in the rhizosphere affect the attainable yield at significant level (Cook, 2007). Plant-growth promoting rhizobacteria (PGPR), in conjunction with efficient *Rhizobium* (*Bradyrhizobium*) can affect the growth and nitrogen fixation in pigeon pea by inducing the occupancy of introduced *Bradyrhizobium* in the nodules of the legume (Tilak *et al.,* 2006).

Availability of phosphate in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes (Haque and Dave 2005). Phosphate solubilization by Phosphate solubilization bacteria (PSB) strains is associated with the release of low molecular weight organic acids mainly gluconic and ketogluconic (Kim *et al.,* 1997), which through their carboxyl and hydroxyl groups chelate the cations bound to phosphate, therefore converting it into soluble forms. The production of gluconic acid also reported to be the most frequent agent of mineral phosphate solubilization (Shahab and Ahmed; 2008).

Phosphorus (P) deficiency in soil can severely limit plant growth productivity, particularly in legumes, where both the plants and their symbiotic bacteria are affected, and this may have a deleterious effect on nodule formation, development and function (Alikhani *et al*., 2006). Besides, symbiotic nitrogen fixation, a few strains or species of *Rhizobium* are involved in phosphate solubilization also (Deshwal *et al.,* 2003). But studies on phosphate solubilizing ability of *Rhizobium* strains are very limited (Halder *et al.,* 1990; Haldar *et al.,* 1991; Halder and Chakrabarty, 1993; Rivas *et al.,* 2006; Daimon *et al.,* 2006). The main advantage of using rhizobia as a phosphate-solubilizing microorganism will be their beneficial nutritional effect resulting both from phosphate mobilization and nitrogen fixation (Peix *et al.,* 2001). The application of Phosphate solubilization bacteria (PSB) strains in agricultural practice would not only offset the high cost of phosphate fertilizers but would also mobilize insoluble phosphorus available in the fertilizers and soils to which they are applied. Bacterial genera like *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Bhattacharya and Jha, 2012). Exploration & exploitation of the microbial community in the rhizosphere is very crucial to study & understand their biology, PGPR traits, ecological interaction with the host plant & population dynamics. Keeping in view the above points, the present study was made with 13*Bradyrhizobium (*S1 to S14 except S5) and two endophytic (S5 & S15) strains (identified by 16s rRNA sequencing) isolated from root nodules of 15 different locations and were tested for their ability to solubilize tricalcium phosphate (TCP).

## Material and Methods

Root nodule samples of 60 days old pigeon pea plants were obtained from the Begusarai, Vaishali, Samastipur districts of Bihar; Mau, Gazipur, Mirzapur & Varanasi districts of Uttar Pradesh. Root nodule samples were kept in sterile polythene bags and brought to laboratory for isolation of *Rhizobia*. The selective medium yeast extract mannitol agar (YEMA) was used for isolation of rhizobia (Vincent JM 1970) and a pure culture of each isolate was prepared after subculturing on the same medium (YEMA). Pure cultures were authenticated as rhizobia through their nodulating ability on homologous hosts by plant infection tests (Vincent JM 1970) and 16s rRNA sequencing. The phosphatesolubilizing ability of the isolates was tested on Pikovskaya's agar medium (Pikovskaya RI 1948) containing TCP as insoluble phosphate source. a pin point inoculation of the Petri plates was made on plates under aseptic conditions and incubated at 28±2 ºC for 5 days. Formation of zone around the colonies on Pikovskaya's medium indicates the phosphate solubilization ability of the organism. The diameter of the zone of solubilization was measured and expressed in mm (Vazquez *et al.,* 2000). Phosphate SI was determined by measuring both colony and halo zone diameters using Edipremono*et al.,* (1996) formula:

Phosphate SI = (colony diameter + halo zone diameter)/ colony diameter

Quantitative analysis of phosphate solubilization by isolates was determined using Pikovskaya broth. Conical flasks containing Pikovskaya broth were inoculated with separate isolates at 30˚C for four days on a shaker at 150 rpm. Isolates were centrifuged at regular intervals at 5000 rpm for 10 min and available soluble phosphate was measured in supernatants using phosphomolybdate method [Watanabe and Olsen, 1965] spectrophotometrically at 475 nm. Quantification of phosphorous requires the conversion of the phosphorus to dissolved orthophosphate followed by spectrophotometric determination of dissolved orthophosphate. Ammonium molybdate and antimony potassium tartrate react in an acid medium with diluted solutions of orthophosphate to form an intensely colored antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.

## Result and Discussion

The *Bradyrhizobium* and endophytic strains isolated from the root nodules of pigeon pea plants were tested *in-vitro* for phosphate solubilization activity on pikovskaya media. Out of total 15 strains, ten strains tested (S1, S3, S5, S6, S8, S9, S10, S11, S12 and S13) positive for phosphate solubilization ability by making halo zones around colony on medium after 96 hours (Fig. 1). The strains S8, S9, S11 & S13 made larger halo zones in comparison to the other six strains *i.e.,*S1, S3, S5, S6, S10 and S12 which indicated that S8, S9, S11 & S13 had more ability to solubilize phosphate in comparison to other strains. Size of solubilizing zones ranged from 4mm to 32 mm in diameter. The Bradyrhizobium strains S9 (32 mm) was highest effective in phosphate solubilization followed by S8 (29 mm), S11 (22 mm), S13 (21 mm), S1 (18 mm), S5 (12 mm), S3 (12 mm), S12 (6mm), S10 (6mm) & S6 (4 mm) in 96 hours (Table 1, Fig. 1& Fig. 2). The phosphate solubilization index (SI) was calculated in which highest SI was observed for the strains S11 (3.4) & S4 (3.4) followed by S13 (3.1), S9 (3.0), S10 (3.0), S12 (3.0), S1 (2.5), S8 (2.2), S3 (2.2) & S6 (2.1) (Table 1 & Fig. 2). Same experiments with these strains (S1, S3, S5, S6, S8, S9, S10, S11, S12 and S13) were performed in pikovskaya broth and optical density was measured at 475 nm wavelength after 24 to 96 days. The result showed the increase in OD value with increase in duration for all the strains. Highest OD was observed for the strain S11 (1.984) followed by S4 (1.824), S13 (1.759), S9 (1.747), S10 (1.554), S12 (1.421), S1 (1.401), S8 (1.332), S3 (1.366) &S6 (1.246) after 96 hours (Table 2 & Fig. 3). It was also observed that there is perfect correlation between phosphate solubilization efficiency on solid and liquid medium *i.e.,*result of phosphate solubilization index obtained through pikovskaya agar plate was exactly similar to that obtained through liquid medium.

This study showed that out of 15 bacterial strains tested for phosphate solubilizing ability, only nine *Bradyrhizobium* strains (S1, S3, S6, S8, S9, S10, S11, S12 and S13) and one endophytic strain (S5) showed their ability to solubilize the phosphorus.

From this study it is clear that phosphate solubilization is not a widespread character among rhizobia, and not all the Rhizobium strains, even from a single host (P. pea), exhibit phosphate solubilization. Hence, it is necessary to study the phosphate-solubilizing activity of a large number of *Rhizobium* strains from a single host. Furthermore, the Rhizobium strains showing phosphate solubilization could be used for understanding the mechanism of phosphate acquisition in host plants from which they were isolated.When pure cultures of PSBs are added to the soil, phosphate nutrition is increased to the plant resulting in increased phosphate availability to the plants (Gerretsen 1948). Several studies have shown that phosphate solubilizing microorganisms solubilizes the fixed soil phosphorus and applied phosphates which results in higher crop yields (Gull *et al*., 2004; Zaidi 2009). As per several reports, PSB applied with PGPR could significantly reduce P fertilizer application by 50% without any significant loss in crop yields (Jilani *et al*., 2007). Halder *et al.* (1990) reported the solubilization of rock phosphate by *Rhizobium & Bradyrhizobium.* This study showed that the extent of phosphate solubilization by the root-nodule bacteria is very comparable to that by other bacteria reported. Phosphate-solubilizing microbes helps to offset the high cost of phosphatic fertilizers by mobilizing insoluble phosphorus applied as fertilizers and present in soils. Thus, application of such organisms' multiple plant growth-promoting activities can increase the productivity of crops including legumes significantly for sustaining crop production with optimized P fertilization. It is eco-friendly as well as viable alternative. Inoculation of Phosphate-solubilizing bacteria either alone or in synergism with other PGPRs has been shown to enhance the performance of crop plants including legumes (Shaharoona *et al*., 2008; Wani *et al*., 2007a) and therefore, PSB could be used as biofertilizer for improving the productivity of crops plants in different agro-ecological niches. Thus, the identified PSB bacterial strains from this study can be developed as the bioinoculant or bio fertilizer which may help in the development of ecofriendly and sustainable agriculture.

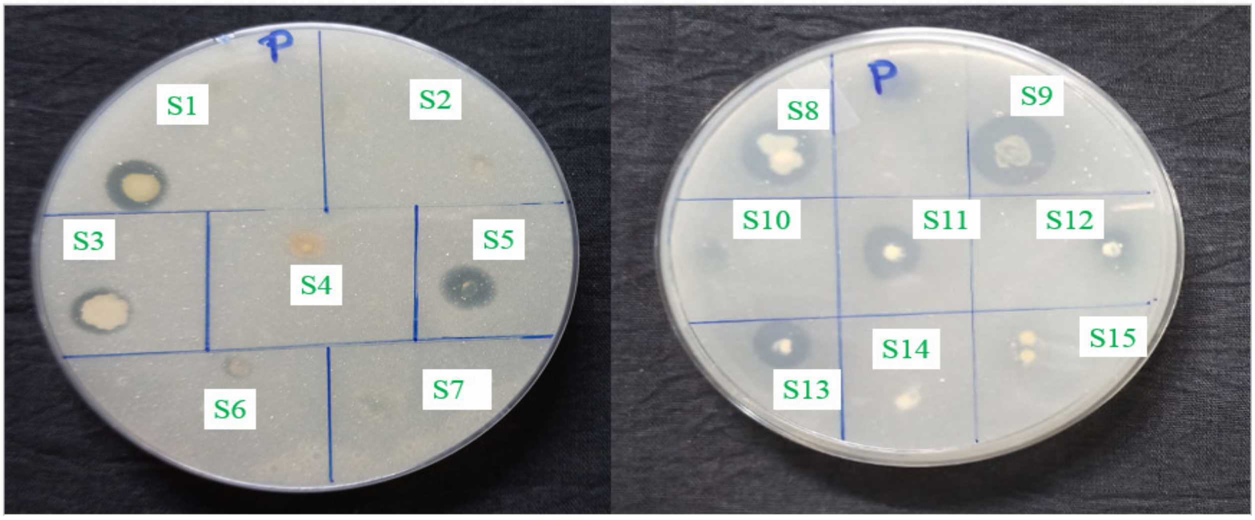

**Fig. 1 :** Determination of phosphate solubilisation activity of pigeon pea *Bradyrhizobium* strains

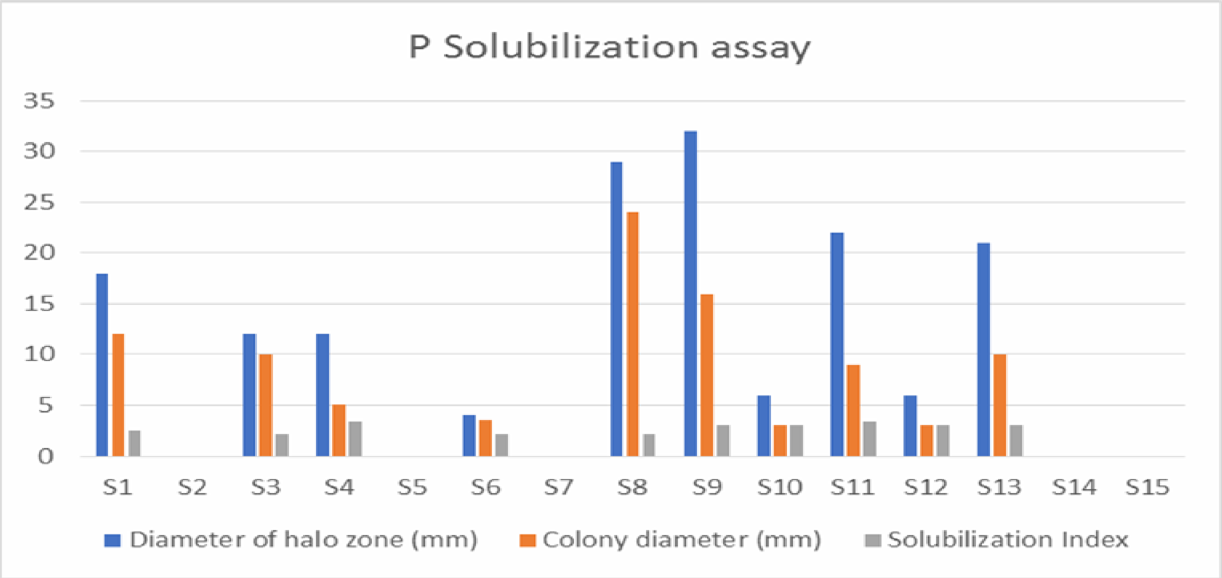

**Fig. 2 :** Phosphate solubilization ability of different bacterial strains on Pikovskaya's agar

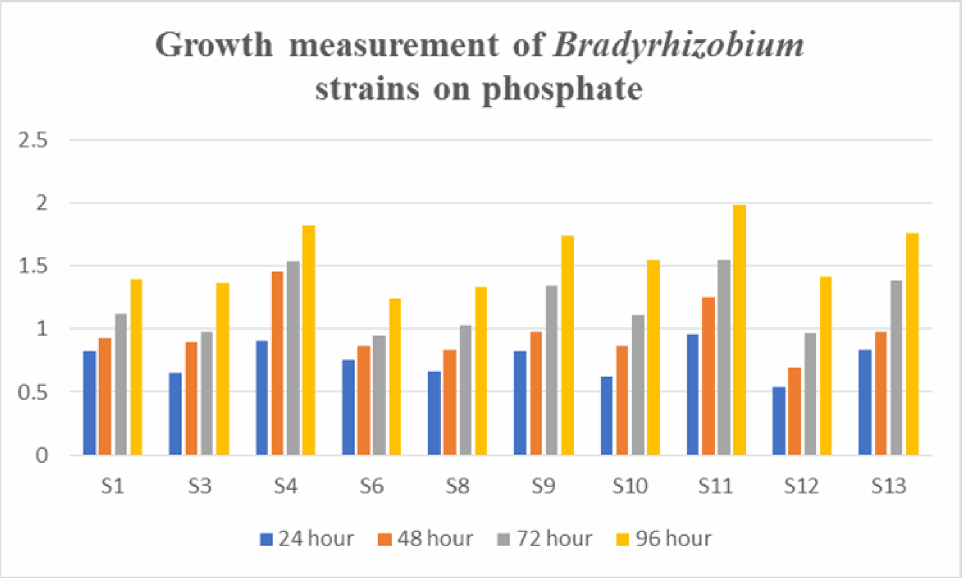

**Fig. 3 :** Phosphate solubilization ability of different bacterial strains on liquid medium at different time interval

**Table 1 :** Solubilization of tricalcium phosphate by bacterial isolates on Pikovskaya's agar

| Strains | Diameter of halo zone (mm) | Colony diameter (mm) | Solubilization Index |
|---|---|---|---|
| S1 | 18 | 12 | 2.5 |
| S2 | 0 | 0 | 0.0 |
| S3 | 12 | 10 | 2.2 |
| S4 | 12 | 5 | 3.4 |
| S5 | 0 | 0 | 0.0 |
| S6 | 4 | 3.5 | 2.1 |
| S7 | 0 | 0 | 0.0 |
| S8 | 29 | 24 | 2.2 |
| S9 | 32 | 16 | 3.0 |
| S10 | 6 | 3 | 3.0 |
| S11 | 22 | 9 | 3.4 |
| S12 | 6 | 3 | 3.0 |
| S13 | 21 | 10 | 3.1 |
| S14 | 0 | 0 | 0.0 |
| S15 | 0 | 0 | 0 |

**Table 2 :** Solubilization of tricalcium phosphate by bacterial isolates on liquid medium

| | Incubation period (hours) (OD Value) | | | |
|---|---|---|---|---|
| Strains | 24 hours | 48 hours | 72 hours | 96 hours |
| S1 | 0.826 | 0.924 | 1.122 | 1.401 |
| S3 | 0.651 | 0.895 | 0.983 | 1.366 |
| S4 | 0.912 | 1.462 | 1.539 | 1.824 |
| S6 | 0.754 | 0.869 | 0.947 | 1.246 |
| S8 | 0.658 | 0.839 | 1.026 | 1.332 |
| S9 | 0.825 | 0.978 | 1.346 | 1.747 |
| S10 | 0.621 | 0.865 | 1.112 | 1.554 |
| S11 | 0.954 | 1.254 | 1.548 | 1.984 |
| S12 | 0.542 | 0.698 | 0.973 | 1.421 |
| S13 | 0.841 | 0.982 | 1.385 | 1.759 |

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