Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 2566-2570
Received: 15-06-2020
Accepted: 19-07-2020

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Screening of efficient phosphate solubilising bacteria from different chickpea growing areas of Karnataka

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DOI: https://doi.org/10.22271/phyto.2020.v9.i5aj.12728

Abstract
Different Chickpea growing areas of Karnataka like Arasikere, Chamarajanagara, Mysore and Devanahalli were selected for the isolation and screening of phosphate solubilising bacteria. Totally 24 isolates were isolated from four soil samples of different Chickpea growing areas. Based on zone of solubilization ten isolates were selected to investigate their efficiency to solubilize insoluble phosphate in Pikovskaya's agar media under *in vitro* conditions. Among 10 isolates ACP-3 recorded higher PSE of 188.8% followed by ACP-2 (150.0%) and CCP-2 (145.4%). All the 10 isolates were tested for their ability to release percent Pi from tricalcium phosphate (TCP) in the Pikovskaya's broth media. Highest percent Pi released from TCP by the isolate ACP-3 (17.36%) followed by CCP-2 (14.14%). Same isolates were also tested for pH change in the Pikovskaya's broth at different days of intervals. ACP-3 recorded least pH which shows maximum decrease in pH of Pikovskaya's broth followed by ACP-2 and CCP-2. All the three experiments result shows that among the 10 isolates ACP-3 was found to be most efficient phosphate solubilising bacteria and this isolate was identified as *Bacillus licheniformis* from 16srRNA sequencing.

Keywords: Phosphate solubilising bacteria (PSB), Pi released, phosphate solubilization efficiency

Introduction
Phosphorus (P) is the key element in the nutrition of plants next to the nitrogen. Commonly known as the 'energy currency' as it is associated with the several vital functions of life. P is necessary for growth of all the forms of life on the planet earth including plants, animals and microorganisms as a component of nucleic acids, phospholipids that compose cellular membrane, ATP and ADP molecules. Phosphorus plays important role in all the metabolic process including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration. For the food production in India one of the major constraints is the poor P status of the soil (Dotaniya *et al.*, 2013) [4].

Phosphorus exists in nature in a variety of organic and inorganic forms. Although P is abundant in soils in both organic and inorganic forms, its availability is restricted to plants as it occur mostly in insoluble forms (Pradhan and Sukla, 2005) [15]. Since P deficiency is the most important key limiting factor restricting the plant growth, chemical phosphatic fertilizers are widely used to increase crop yields, where soluble forms of P fertilizer used are easily precipitated as insoluble form, which leads to excessive and repeated application of P fertilizers (Alam *et al*. 2002) [1]. A large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized and becomes unavailable to crop plants.

A large number of microorganisms present in the rhizosphere are known to solubilize insoluble phosphorus (Ranadev *et al.*, 2019) [16] and make available to the plants and are called as phosphorus solubilizing microorganisms (PSM) which include phosphorus solubilizing bacteria (PSB) and phosphorus solubilizing fungi (PSF). Phosphorus solubilizing activity is carried out by a large number of saprophytic bacteria and fungi through various microbial processes or mechanisms including organic acid production and proton extrusion (Karpagam and Nagalakshmi, 2014) [11]. Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelates cations and decreases the pH. The PSB dissolves the soil P through the production of low molecular organic acids like gluconic and ketogluconic acids. P solubilization ability of PSB has direct correlation with pH of the medium. The use of phosphate solubilizing bacteria as inoculants simultaneously increases P uptake by the plant and crop yield. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Hilda and Reynaldo., 1999) [7].

Consequently, chemical fertilizers are frequently applied during crop planting, but its regular use is costly and produces undesirable environmental impacts, such as soil and water contamination. Therefore, P is often regarded a limiting nutrient in agricultural soils (Guinazu *et al.*, 2010; Yu *et al.*, 2011) [6, 21].

In the recent years, the cost of chemical fertilizers especially phosphatic fertilizers is increasing which is away from reach of small farmers in the developing countries like India. This has led to the finding of alternative ways for supplying the P to the crop plants in a cost effective way. One such way is development and use of phosphorus solubilizing biofertilizers. By this, we can supply low grade insoluble rock phosphate in combination with phosphorus solubilizing microorganisms which is an alternative cost effective way against costly chemical phosphatic fertilizers like single super phosphate. PSB such as *Bacillus megaterium*, *Bacillus circulans*, *Pseudomonas striata* are effective biofertilizers. In this regard the present study was taken up to isolate the efficient PSB from different chickpea growing areas of Karnataka.

Materials and Methods

Isolation of phosphate solubilizing bacterial isolates

Soil samples were collected from different Chickpea growing areas of Karnataka like Arasikere, Chamarajanagara, Mysore and Devanahalli. Pikovskaya's medium (Pikovskaya, 1948) was used for the isolation of phosphorus solubilizing bacteria. PSB were isolated from each sample by serial dilution and spread plate method. After 2-3 days of incubation, colonies forming halo zones were selected and sub-cultured for further study.

Screening of efficient phosphate solubilizing bacterial isolates

Phosphate solubilization ability of each PSB isolate was determined by measuring the zone of P solubilization on the Pikovskaya's agar medium and by estimating percent P solubilization in Pikovskaya's broth medium and pH change in the culture media due to the P solubilization.

Zone of solubilization/Phosphate solubilization efficiency

For determining zone of P solubilization, Pikovskaya's agar medium was poured on the Petriplates, after solidification 10 µl broth culture was spot inoculated on the plates. The plates were incubated at 28 ± 2 °C for about 3-5 days and solubilization zone was observed around the colony. P solubilization diameter was recorded and phosphate solubilization efficiency was calculated by using the following formula.

PSE = Solubilization diameter/Growth diameter × 100

Quantitative estimation of Pi released from tricalcium-phosphate (TCP)

The isolates showing zone of solubilization on Pikovskaya's agar were further examined for their ability to release Pi from TCP in broth medium. One ml of culture of isolate incubated overnight was inoculated to 50 ml of Pikovskaya's broth. The inoculated flask was incubated for two weeks at 28 ± 2 °C. The amount of Pi released in the broth was estimated at 5, 10, and 15 days of incubation from triplicate flask at each stage in comparison with a set of uninoculated controls. The broth cultures were centrifuged at 10,000 rpm for 10 minutes in a centrifuge to separate the supernatant from the cell growth and insoluble phosphate. The available P content in the supernatant was estimated by phosphomolybdic blue color method of Jackson (1973) [9].

One ml of the culture supernatant was taken in 50 ml volumetric flasks to which 10 ml of chloromolybdic acid was added and mixed thoroughly. The volume was made up to approximately three fourth with distilled water and 0.25 ml chlorostannous acid was added and the volume was made to 50 ml with distilled water and mixed thoroughly. After 15 minutes, the blue color developed was read in spectrophotometer at 610 nm using a reagent blank. Simultaneously, a standard curve was prepared using various concentrations of standard 2 ppm KH_2PO_4 solution. The amount of phosphorus solubilized by the isolates was calculated from the standard curve.

pH Change

Three days old test cultures were added to sterile Pikovskaya's broth medium in conical flask and kept on shaker for seven days. Sterile uninoculated medium served as control. Initial pH was recorded and change in pH was noted at two days interval for one week by digital pH meter (Alam *et al.*, 2002) [1].

Results and Discussion

Phosphate solubilization efficiency of the PSB isolates

Ten isolates examined for their ability to solubilize TCP on Pikovskaya's agar medium supplemented with TCP as insoluble P source. All the isolates showed zone of solubilization. The isolate ACP3 showed the highest P solubilization of 188.8% followed by ACP-2 (150.0%) and CCP-2 (145.4%) where as lowest solubilization was found in MCP-2 (120.0%) and the data is presented in Table 1.

The per cent P solubilizing efficiency (PSE) ranged from 120 per cent to 188.8 per cent. Where the highest (188.8%) PSE was recorded by the ACP-3 isolate followed by ACP-2 (150%). Similar observations were recorded by Sharma *et al.*, 2007 where the solubilization efficiency of *Psuedomonas fluorescens* and *Bacillus megaterium* were recorded as 200 and 128.57% respectively and also this was conformity with the findings of Guar *et al*, (1973) [5], where *Bacillus megaterium* var. *phosphaticum* recorded maximum percentage of phosphate solubilization.

Table 1: Phosphate solubilization efficiency of the PSB isolates

Sl. No.	PSB isolates	PSE%
1	ACP-1	127.2
2	ACP-2	150.0
3	ACP-3	188.8
4	ACP-4	122.2
5	CCP-1	125.0
6	CCP-2	145.4
7	CCP-3	125.0
8	MCP-1	122.2
9	MCP-2	120.0
10	MCP-3	133.3
11	Control	0.0

Quantitative estimation of Pi released by the PSB isolates

The amount of Pi released from TCP in the Pikovskaya's broth by PSB isolates was studied at 5, 10, and 15 days after incubation (DAI). The results are presented in Table 2. It evident that the amount of Pi released from TCP by PSB isolates was increased with advanced incubation time and was maximum at 15 DAI. The highest per cent Pi released at 15

DAI was found in ACP-3 (17.36%) followed by ACP-2 (15.11%) and CCP-2 (14.14%), whereas lowest solubilization was found in MCP-2 (9.59%).

Table 2: Quantitative estimation of Pi released by the PSB isolates

Sl. No.	PSB isolates	Pi (%) released in the Pikovskaya's broth		
		5 DAI (%)	10 DAI (%)	15 DAI (%)
1	ACP-1	5.31	8.13	12.75
2	ACP-2	7.65	9.73	15.11
3	ACP-3	7.84	10.42	17.36
4	ACP-4	5.83	8.12	12.56
5	CCP-1	4.15	6.24	8.14
6	CCP-2	6.85	9.13	14.14
7	CCP-3	5.43	6.58	11.09
8	MCP-1	5.67	7.04	10.45
9	MCP-2	5.35	6.84	9.59
10	MCP-3	6.12	7.13	11.47
11	Control	0.65	0.96	1.2

The highest (17.36%) per cent Pi released from TCP was recorded by the ACP-3 isolate followed by ACP-2 (15.11%) and CCP-2 (14.14%). The release of P from insoluble phosphates by the bacterial isolates increased with incubation time upto 15 days. In the present investigation, amount of TCP solubilized by PSB isolates ranged from 9.5% to 17.3%. Sen and Paul (1957) [17] reported a solubilization of 4.36 to 9.65 per cent in case of TCP, 0.77 to 2.07 per cent in case of Fe_2PO_3 and 3.62 to 10.82 per cent in case of calcium glycerophosphate by different organisms. However, decreased thereafter, which could be due to nutrient limitation or immobilization of Pi has been reported. In case of phosphate solubilizing bacteria, amount of Pi released from TCP at different incubation period was maximum at 15 DAI (17.47). Similar observation of solubilization of insoluble phosphate by Badar (2006) [2] and Hu *et al.*, (2006) [8] has been made. The results also indicated variability in the amount of Pi released by different isolates. The differential efficiency of bacteria to solubilize insoluble inorganic potassium and phosphate could be due to differences in their ability to release organic acids (Sheng and He. 2006; Liu *et al.*, 2006) [19, 13].

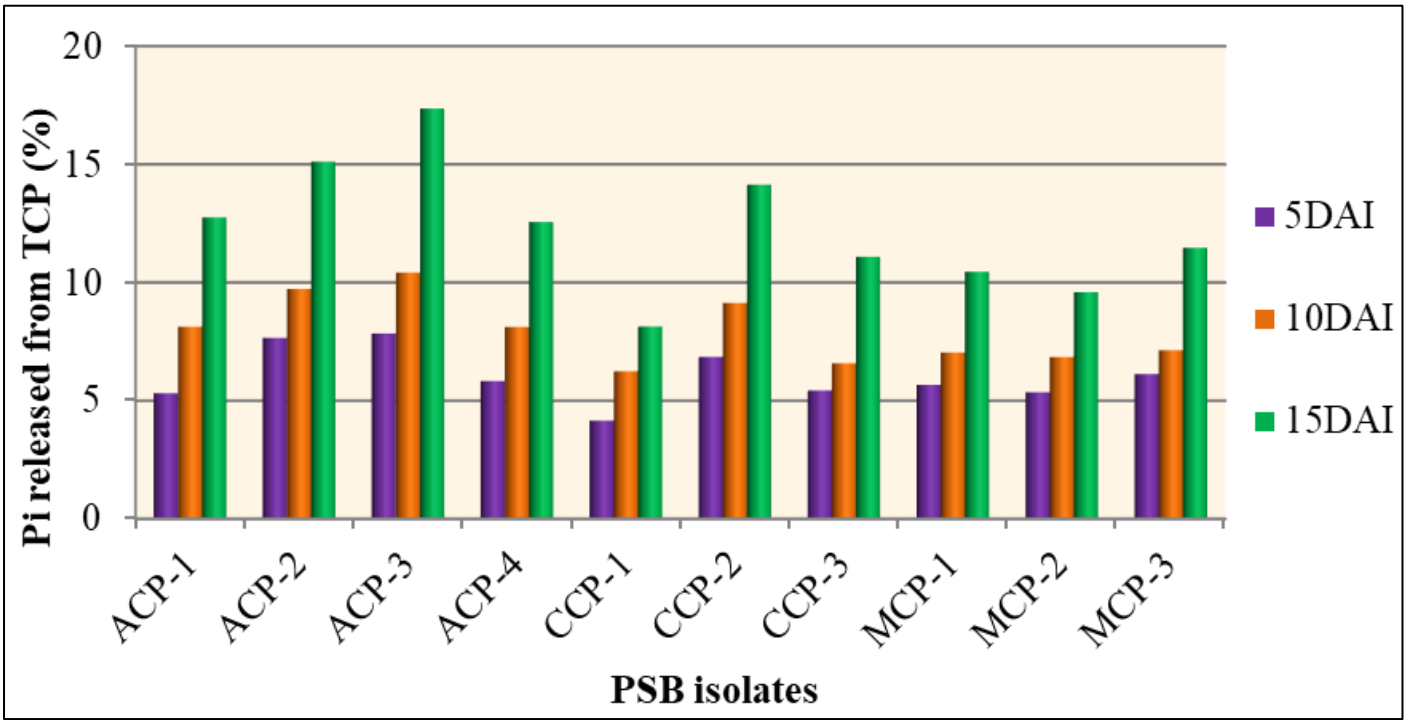

Fig 1: Quantitative estimation of Pi (%) released from TCP by the PSB isolates at different days after incubation (DAI)

Table 3: Effect of phosphorus solubilising bacteria growth on pH of the Pikovskaya's broth medium

Sl. No.	PSB isolates	pH change in the broth medium		
		3 DAI	5 DAI	7 DAI
1	ACP-1	5.77	5.33	4.83
2	ACP-2	5.60	4.85	4.25
3	ACP-3	5.54	4.78	4.13
4	ACP-4	5.84	5.32	4.76
5	CCP-1	5.93	5.46	4.85
6	CCP-2	5.68	4.97	4.32
7	CCP-3	5.9	5.43	4.92
8	MCP-1	5.97	5.64	5.18
9	MCP-2	6.08	5.67	5.25
10	MCP-3	6.33	5.93	5.20
11	Control	6.88	6.65	6.61

The selected ten PSB isolates were tested for change in pH of Pikovskaya's broth during solubilization at different incubation periods. Data on the change in the pH of the broth medium at 3, 5 and 7 DAI by PSB isolates are presented in the Table 3. The change in pH of the medium (initial pH of broth 7.0) was found to decreases with an increase in incubation period and lowest pH was recorded at 7 DAI ranged from 5.25-4.13 pH whereas highest pH was reduced by ACP-3 (4.13) followed by ACP-2 (4.25) and CCP-2 (4.32), whereas lowest solubilization was found in MCP-2 (5.25). Alam *et al.* (2002) [1] reported drop in pH of the medium by PSB isolates ranging from 4.00 to 3.20 at the end of incubation. Similar results were observed by Khalil and Sultan (2000) [10].

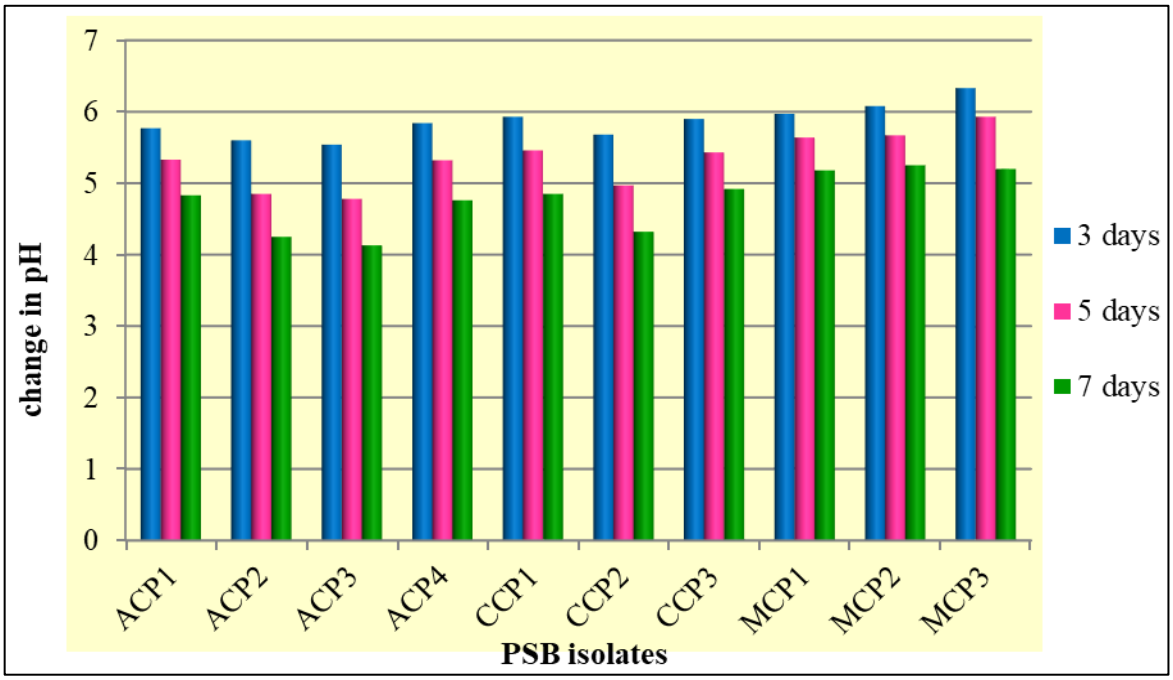

Fig 2: Effect of the selected PSB isolates on pH of Pikovskaya's broth medium at different days aincubation (DAI)

To judge the P solubilizing efficiency of PSB isolates, the phosphorus solubilization efficiency, estimation of percent Pi released and drop in the pH of the broth medium were considered as dependable criteria. Similar emphasis on these parameters was given by Souchie *et al.* (2007) [20] at the time of isolation and initial screening of phosphate solubilizing microorganisms. It was evident from results of the present investigation that PSB isolates differed in their capacity to form the clear zone of P solubilization as well as on the efficiency in terms of percent P released. The findings of the experiment corroborate with results obtained by Kundu *et al.* (2009) [12] where they also classified 193 PSB isolates into five categories based on P- solubilization efficiency and P solubilization potential. The principal mechanism in soil for mineral phosphate solubilization is decrease in soil pH by microbial production of organic acids and mineralization of organic P by acid phosphatase (Bagyaraj *et al.*, 2000) [3].

Conclusion

From this investigation it is found that the soils of chickpea growing areas are naturally colonized by PSB. Among the selected four different regions the efficient phosphate solubilizing bacteria isolates were found in Arasikere soil sample. These phosphatic biofertilizers are eco-friendly and helpful in reducing the cost of chemical fertilizers also prevents adverse effects on environment. Therefore further study has to be conducted to test the effect of efficient PSB isolates on crop growth under green house and field conditions.

Acknowledgement

I express my sincere gratitude to Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru for providing all the facility and it makes me immensely proud and honored to be a student of this prestigious university. I take this opportunity to thank major advisor and chairman of advisory committee Dr. B Narayanaswamy Professor, Dept. of Agril. Microbiology, GKVK UAS Bengaluru and my sincere gratitude and heartfelt respect to the blessings of my parents, support of siblings, friends and all the staff members of the department.

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