Characterization of Mineral Phosphate Solubilizing Gram-positive Bacteria

Srinivasamurthy, R.[*] and Dayamani, K.J. [**]

[*]Jr. Scientific Officer, NCOF, Ghaziabad,
[**]Assistant Professor, College of Horticulture, UHS Campus, GKVK, Bengaluru-65

***Abstract*-** Most agronomic soils contain large reserves of total phosphorus [P], but the fixation and precipitation of P cause P deficiency, and in turn, restrict the growth of crops severely. Phosphorus replenishment, especially in sustainable production systems, remains a major challenge as it is mainly fertilizer-dependent. Though the use of chemical P fertilizers is obviously the best means to circumvent P deficiency in different agro-ecosystems, their use is always limited due to its spiralling cost. A greater interest has, therefore, been generated to find an alternative yet inexpensive technology that could provide sufficient P to plants while reducing the dependence on expensive chemical P fertilizers. Among the heterogeneous and naturally abundant microbes inhabiting the rhizosphere, the phosphate solubilizing microorganisms (PSM) including bacteria have provided an alternative biotechnological solution in sustainable agriculture to meet the P demands of plants. These organisms in addition to providing P to plants also facilitate plant growth by other mechanisms. Despite their different ecological niches and multiple functional properties, P-solubilizing bacteria have yet to fulfill their promise as commercial bio-inoculants. Current developments in our understanding of the functional diversity, rhizosphere colonizing ability, mode of actions and judicious application are likely to facilitate their use as reliable components in the management of sustainable agricultural systems.

***Index Terms*-** Phosphorus solubilization, Bacillus spp, IAA and GA

I. INTRODUCTION

Phosphorous is a major macronutrient for plants growth and development (Fernandez et al., 2007). In most soils, phosphorus is being about 0.05%, of which only 0.1% is available to the plants. Most of the phosphorus fertilizers applied to soil may get fixed because of large reactivity of phosphate (PO_4^{3-}) ions with numerous soil constituents (Rodriguez and Fraga, 1999). The soil microorganisms have enormous potential to release plant available phosphorus from unavailable inorganic and organic phosphorus compounds by process of solubilization (Gyaneshwar et al., 2002). The mineral phosphate solubilization by Gram-negative bacteria and their usefulness in increasing crop growth and yield is well known (Mohi et al., 2000; Bagyaraj et al., 2000; Han and Lee 2005; Almas and Mohammad 2006; Fankem et al., 2008). However, scanty of literature is available in case of Gram-positive phosphorus solubilization bacteria and its effect on crop plants. Hence study was conducted to characterize phosphate solubilization by gram positive bacteria.

II. MATERIALS AND METHODS

Isolation and characterization of P-solubilizing bacterial strains

The rhizosphere soil samples along with roots of different crop plants such as soybean, maize sorghum and cowpea were randomly collected from the main Agricultural Research Station, University of Agricultural Sciences, Dharwad. These soil samples were screened for isolation of phosphate solubilizing Gram-positive bacteria on Pikovskaya's agar medium containing tricalcium phosphate (TCP). Plates were incubated at 28 ± 2°C for 72 hrs. Colonies that gave distinct clear circular zone around the colony, which indicated solubilization of phosphorus. These colonies were purified and maintained on Pikovaskay's agar slants for further identification and characterization. During present investigation total of 75 bacterial strains were examined for phosphorus solubilization activity. Out of 75 bacterial strains, thirty three were isolated from the soil samples and fourteen bacterial strains were taken from Department of Microbiology and twenty seven strains were taken from Department of Biotechnology, University of Agricultural Sciences, Dharwad, which were identified up to generic level based on morphological and biochemical characteristics (Bergyey's Manual of Determinative Bacteriology). Apart from above bacterial collections, standard phosphorus solubilizing bacteria *Bacillus polymyxa* was used in all the experiments as a positive control. All the phosphate solubilizing bacteria were initially tested for their efficiency to release phosphorus by inoculating them into the Pikovskaya's broth medium (100ml) containing insoluble sources of phosphorus (Tricalcium phosphate (TCP)) fallowed by incubation at 28°C for 15days. The amount of phosphate solubilization was estimated by the phosmomalybdate blue color method (Jackson, 1973). Twenty three Gram-positive bacteria which released high amount of Pi in Pikovaskay's broth were further selected and tested for their ability solubilize rock phosphate as described above. These twenty three selected strains were also tested for its potential to produce plant growth promoting substances like IAA and GA by the method proposed by Gorden and Paleg (1957). The change in pH of each broth culture filtrates at 15 days after inoculation (DAI) at the time of Pi estimation was examined using digital pH meter.

III. RESULTS AND DISCUSSION

Phosphorus solubilization, change in pH, production of IAA and GA in broth media

Total of seventy five Gram-positive bacteria were examined including reference strain *B. polymyxa* for their activity to release Pi in Pikovskay's broth containing tri-calcium phosphate (TCP) medium and simultaneously measured change in pH of culture filtrates. It was found that, the great variability among bacteria to solubilization of phosphorus from insoluble source media (Table 1). The 23 bacterial strains out of total strains (75) released high amount of Pi from TCP were selected and these bacterial strains of interest were tested for it's ability to solubilize rock phosphorus and plant growth promoting substances (IAA and GA) production.

There was a great variation in activity phosphate solubilization of isolated strains has been recorded (Table 1). The reference strain *B. polymyxa* released 8.40% solubilized phosphorus from TCP. Some isolated strains had indicated much more activity for phosphate solubilization as compared to reference strain. An isolate *Bacillus spp* (B3) had been produced 7.95% more solubilized phosphorus. Similarly other bacterial strains *B. thuriningenesis* T1, T6 and *Arthrobacter spp* (A1) have been indicated 6.92 %, 5.36 % and 3.22 % respectively as compared to standard strain *B. polymyxa.* The change in pH of the culture filtrates was measured simultaneously with percent Pi estimation by using digital pH meter. The initial pH of 7.0 was maintained at the time of inoculation. The results showed decrease in pH of all the culture filtrates after 15 DAI (Days after inoculation) as compared to initial pH of the broth medium. The drop in pH ranged from 1.31 to 3.46 units at 15 days after inoculation was observed (Table 1). The media inoculated with *B. thuringiensis* PP2 recorded maximum drop in the pH (3.46 units) at 15 days after inoculation. The reference strain *B. polymyxa* recorded 2.86 units drop in the pH of the TCP medium at 15 days after inoculation.

On the other hand, above strains inoculated in broth media supplemented with rock phosphate have shown almost similar pattern of phosphate solubilization (Table 2). Reference strain solubilized 4.27 % of rock phosphate. Among all isolates studied some of them were found active phosphorus solubilization. The isolated strains *Arthrobacter spp* (A1), *Bacillus spp* (B3, B7 and EB10), *B. thuringiensis* (T1, T6, T7, T4 and R6) and unidentified strain 121 were found more active for the solubilization of phosphorus from rock phosphate as compared to reference strain (Table 2). In general *Bacillus spp* were shown more active phosphorus solubilization as compared to other strains.

The change in pH of broth medium containing rock phosphate was also measured at 15 DAI. The drop in pH ranged from 1.35 to 2.91 units at 15 DAI (Table 3). The medium inoculated with *B. thuringiensis* T1 showed maximum drop in the pH (2.91 units) closely followed by *Bacillus spp* B7 (2.65 units) and *B. thuringiensis* T6 (2.6 units). The similar trend of decreasing pH was also observed over a period of incubation with phosphate solubilizing bacteria (Sadaf and Nuzhat, 2008). These results had indicated great variation among Gram-positive bacteria to solubilize the insoluble phosphorus with concomitant decrease in pH of broth medium. The results were indicated acidification of the broth medium is one of the mechanism involved by phosphate solubilizing bacteria. It could be due to production of organic acids by phosphate solubilizing bacteria (Rodriguez et al., 2006; Fankem et al., 2008). This also confirms the microbial activity and its metabolites play a major role in phosphorus solubilization.

The Gram-positive P-solubilizing bacteria in addition also have the ability to produce plant growth promoting substances like IAA and GA at varying concentrations under *in vitro* conditions. Except *Arthrobacter spp* all the other Gram-positive bacteria produced IAA and GA in the range of 0.05 to 0.554 µg ml^{-1} and 0.002 to 0.128 µg ml^{-1} broth. Maximum amount of IAA (0.554 µg ml^{-1}) and GA (0.128 µg ml^{-1}) were produced by *Bacillus spp* (B3). Other strains such as, *B. thuringiensis* T1 had produced 0.560 µg ml^{-1} IAA and 0.120 µg ml^{-1} GA (Table 4). Variation in the amount of IAA and GA production by different P-solubilizing Gram-positive bacteria had probably indicated the metabolic variability among the P-solubilizing bacteria (Defrietas et al., 1997).

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AUTHORS

First Author – Dr. Srinivasamurthy R, Jr. Scientific Officer, NCOF, Ghaziabad, srinrcpb@gmail.com

Second Author – Dr. Dayamani, K.J., M.Sc (Agri.) Ph.D, Assistant Professor, College of Horticulture, UHS Campus, GKVK, Bengaluru-65,.

Correspondence Author – Dr. Dayamani, K.J., gemdaya@gmail.com, gemdaya@yahoo.co.in, Phone: +91 7259552631

Table 1. Percent Pi release from Pikovsakay's broth medium containing tricalcium phosphate (TCP) and change in pH of broth medium at 15 days after inoculation by Gram-positive bacteria.

Sl.No	Isolate No.	% Pi release	pH	Sl. No.	Isolate No.	% Pi release	pH
1.	A1	2.86	6.08	39.	PP1	6.24	4.92
2.	A2	2.13	6.50	40.	PP2	7.24	4.70
3.	A3	1.81	6.67	41.	PP3	7.31	5.00
4.	A4	1.13	5.80	42.	PP4	7.01	5.10
5.	A5	1.18	5.86	43.	PP5	5.38	4.84
6.	A6	0.99	5.34	44.	PP6	4.13	4.85
7.	A7	0.99	6.20	45.	PP7	4.09	4.87
8.	A8	0.99	5.36	46.	PP8	3.88	4.95
9.	A9	1.97	5.67	47.	PP9	4.16	4.98
10.	A10	1.11	5.78	48.	PP10	3.98	4.99
11.	B1	0.98	5.33	49.	PP11	4.59	4.83
12.	B2	1.13	5.40	50.	PP12	4.46	4.80
13.	B3	5.50	5.45	51.	PP13	3.98	5.00
14.	B4	0.97	4.89	52.	M1	1.30	5.05
15.	B5	1.18	6.15	53.	M2	1.06	5.55
16.	B6	1.25	5.50	54.	M3	1.28	6.04
17.	B7	1.89	5.60	55.	M4	1.55	6.10
18.	B8	1.74	5.40	56.	M5	0.96	6.59
19.	B9	4.12	5.26	57.	M6	1.19	6.15
20.	B10	1.81	5.45	58.	M7	2.14	5.76
21.	EB3	0.92	6.15	59.	M8	9.80	6.20
22.	EB9	0.93	6.59	60.	M9	9.52	5.92
23.	EB10	3.00	5.26	61.	M10	8.62	6.65
24.	49	9.18	4.80	62.	11	10.15	6.40
25.	T1	14.92	4.84	63.	131	10.02	4.87
26.	T2	6.12	4.92	64.	16	8.43	5.26
27.	T3	8.13	4.67	65.	94	4.54	5.58
28.	T4	11.17	4.80	66.	98	3.64	5.73
29.	T5	10.81	4.42	67.	81	10.43	6.59
30.	T6	13.76	4.74	68.	121	11.16	6.27
31.	T7	12.77	5.84	69.	104	8.30	6.15
32.	T8	6.12	5.91	70.	124	11.28	5.32
33.	R1	4.72	4.91	71.	100	8.28	5.58
34.	R2	8.86	5.71	72.	93	10.15	6.10
35.	R3	5.65	4.70	73.	105	11.28	4.59
36.	R4	4.64	4.90	74.	47	7.85	5.50
37.	R5	10.01	4.90	75.	*B. ploymyxa*	8.40	5.84
38.	R6	10.45	4.92				

Table 2. Percent Pi release from Pikovsakay's broth medium containing rock phosphate (RP) as a phosphorus source and production of growth-promoting substances (IAA and GA) by efficient Gram-positive bacteria.

Isolate No.	% Pi release	Change in pH	IAA ($\mu g\ ml^{-1}$)	GA ($\mu g\ ml^{-1}$)
Arthrobacter spp.A1	4.90	4.58	-	-
Arthrobacter spp.A2	4.73	5.47	-	-
Bacillus spp. B3	7.76	4.54	0.554	0.128
Bacillus spp. B7	6.10	4.35	0.460	0.080
Bacillus spp. EB10	5.89	4.53	0.434	0.102
*Bacillus thuringiensis*T1	6.39	4.09	0.560	0.120
Bacillus thuringiensis T5	4.28	5.30	0.485	0.095
Bacillus thuringiensis T6	5.35	4.40	0.380	0.076
Bacillus thuringiensis T7	5.15	4.96	0.348	0.069
Bacillus thuringiensis T4	5.76	4.48	0.370	0.069
Bacillus thuringiensis R5	4.28	5.40	0.321	0.025
Bacillus thuringiensis R6	4.82	4.81	0.294	0.017
Bacillus spp.49	3.46	5.57	0.316	0.034
Bacillus polymyxa (*B. p*).	4.27	5.14	0.381	0.104
Micrococcus spp. M7	2.24	5.17	0.132	0.003
Micrococcus spp. M8	3.56	5.40	0.212	0.004
Micrococcus spp. M9	3.53	5.65	0.051	0.012
Micrococcus spp. M10	3.21	5.12	0.147	0.16
Micrococcus spp. 11	3.43	5.48	0.205	0.025
Micrococcus spp.131	3.86	5.36	0.160	0.050
Micrococcus spp.16	3.89	5.12	0.184	0.032
Unidentified 121	4.58	5.25	0.088	0.024
Unidentified 100	3.20	4.85	0.434	0.068
Unidentified 98	3.27	4.84	0.181	0.067

***** DAI : Days after inoculation, *RP : Rock phosphate, ***** IAA: Indole Acetic Acid
***** GA: Giberllic Acid