



Original Paper

Phosphate solubilization activity of rhizobia native to Iranian soils

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Abstract

Agricultural soils in Iran are predominantly calcareous with very low plant available phosphorus (P) content. In addition to their beneficial N₂-fixing activity with legumes, rhizobia can improve plant P nutrition by mobilizing inorganic and organic P. Isolates from different cross-inoculation groups of rhizobia, obtained from Iranian soils were tested for their ability to dissolve inorganic and organic phosphate. From a total of 446 rhizobial isolates tested for P solubilization by the formation of visible dissolution halos on agar plates, 198 (44%) and 341(76%) of the isolates, solubilized Ca₃(PO₄)₂ (TCP) and inositol hexaphosphate (IHP), respectively. In the liquid Sperber TCP medium, phosphate-solubilizing bacteria (*Bacillus* sp. and *Pseudomonas fluorescens*) used as positive controls released an average of 268.6 mg L⁻¹ of P after 360 h incubation. This amount was significantly ($P < 0.05$) higher than those observed with all rhizobia tested. The group of *Rhizobium leguminosarum* bv. *viciae* mobilized in liquid TCP Sperber medium significantly ($P < 0.05$) more P (197.1 mg L⁻¹ in 360 h) than other rhizobia tested. This group also showed the highest dissolution halo on the TCP solid Sperber medium. The release of soluble P was significantly correlated with a drop in the pH of the culture filtrates indicating the importance of acid production in the mobilization process. None of the 70 bradyrhizobial isolates tested was able to solubilize TCP. These results indicate that many rhizobia isolated from soils in Iran are able to mobilize P from organic and inorganic sources and this beneficial effect should be tested with crops grown in Iran.

Introduction

Microorganisms play an important role in effecting the availability of soil P to plant roots, and increasing P mobilization in soil, though the development of effective microbial inoculants remains a major scientific challenge (Richardson, 2001). Agricultural soils in Iran are predominately calcareous and are characterized by a high pH and low amounts of plant available phosphorus (P). The P deficiency can severely limit plant growth and productivity, in particular 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 (Robson et al., 1981). Up to 75% of the soluble P fertilizers added to crops may be converted to sparingly soluble forms by reacting with the free Ca²⁺ ions in high pH soils or with Fe³⁺ or Al³⁺ in low pH soils (Goldstein, 1986). Organic P represents from 50% to 80% of the total soil P, and most plants are unable to utilize these sources of P (Richardson, 2001). Several bacterial and fungal species are phosphate-solubilizing microorganisms (PSM) and evaluation of their potential to mobilize soil P has been the subject of intensive investigations

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(Rodriguez and Fraga, 1999; Whitelaw, 2000). Rhizobia, the beneficial N₂-fixing symbiotic partners of legumes, like other plant growth promoting rhizobacteria (PGPR), are also able to colonize the roots of non-legumes (Chabot et al., 1996; Schloter et al., 1997) and stimulate plant growth (Antoun et al., 1998; Yanni et al., 2001). Rhizobia are able to solubilize both organic (Abd-Alla, 1994) and inorganic phosphates (Antoun et al., 1998). The main advantage of using rhizobia, as PSM will be their dual beneficial nutritional effect resulting both from P mobilization and N₂-fixation (Peix et al., 2001) and their well-documented synergistic interactions with arbuscular mycorrhizal fungi (Barea et al., 2002).

The current study was designed to determine the ability of 446 strains of rhizobia to mobilize inorganic and organic P, in order to identify strains with high activity to be tested as PGPR with crops cultivated in Iran.

Material and methods

The rhizobia

The 446 isolates of rhizobia tested in this study belong to the following groups:

Bradyrhizobium sp. (13); *Bradyrhizobium japonicum* (57); *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (83); *Sinorhizobium meliloti* (168); *Rhizobium leguminosarum* bv. *phaseoli* (57); *Rhizobium leguminosarum* bv. *trifolii* (9) and *Rhizobium leguminosarum* bv. *viciae* (59). All of the isolates originated from fields under legume cultivation in different parts of Iran, and several isolates belong to the Soil and Water Research Institute of Iran. The selective medium, yeast extract mannitol agar (YMA) with congo red (Vincent, 1970), was used for isolation of rhizobia and a pure culture of each isolate was prepared after sub-culturing on the same medium. Pure cultures were authenticated as rhizobia through laboratory procedures and plant infection tests described by Somasegaran and Hoben (1994).

Inocula preparation

In order to prepare fresh inocula containing the same number of bacterial population for all rhizobia under study, a colony of each isolate was

transferred to a 100 mL Erlenmeyer flask containing 15 mL of yeast extract mannitol broth (YMB). Inoculated flasks were incubated at 27 °C on a rotary shaker (100 rpm) for 96 h. All bacterial suspensions were adjusted to approximately 5×10^8 cfu mL⁻¹, with a sterile 0.5% NaCl solution and by using standard curves relating numbers of bacteria (cfu) to optical densities measured with a spectrophotometer at 570 nm.

Phosphate solubilization in solid media

The basal Sperber (1958) medium used contained (in g L⁻¹ of distilled water): glucose 10.0, yeast-extract 0.5, CaCl₂ 0.1, MgSO₄·7H₂O 0.25 and agar 15.0. The medium was supplemented with 2.5 g L⁻¹ of Ca₃(PO₄)₂ (TCP) or inositol hexaphosphate (IHP) as P source to appraise the ability of the strains to mobilize respectively inorganic or organic P sources. The pH of the medium was adjusted to 7.2 before autoclaving. The media were distributed in 9 cm diameter Petri plates and marked in four equal parts after solidification. Using the drop plate method, each part was inoculated with 7 μL of inocula. All tests were performed with four replications. Inoculated plates were incubated in dark at 27 °C and the diameter of clear zone (halo) surrounding the bacterial growth as well as the diameter of colony were measured after 10, 20 and 30 days. All assays were replicated four times and the results are shown as the ratio of halo/colony.

Phosphate solubilization in liquid medium

On solid media three isolates of *Rhizobium leguminosarum* biovar *phaseoli*, 12 of *R. leguminosarum* biovar *viciae*, 69 of *Sinorhizobium meliloti*, and 64 of *Mesorhizobium ciceri* and *M. mediterraneum* produced large halo zones (ratio of halo diameter/colony diameter >1.2), and were used to measure P solubilization in liquid medium. The 70 bradyrhizobia used were unable to solubilize TCP on the solid medium and were also tested in liquid medium. The following P-solubilizing bacteria isolated from Iranian soils were used as positive controls: an isolate of *Bacillus* sp., and three *Pseudomonas fluorescens* isolates. Erlenmeyer flasks (200 mL) containing 90 mL of the liquid Sperber medium were inoculated with 200 μL of bacterial suspension (5×10^8 cfu mL⁻¹).

The flasks were incubated on rotary shaker (120 rpm) at 27 °C. After 72, 120, 240 and 360 h of incubation, aliquots of cultures were aseptically taken from each flask. The supernatant was separated from the bacterial cells by successive filtration through Whatman paper # 42 followed by 0.2 μm Millipore membrane and was used for the determination of the pH and the soluble P released into the solution. P was measured with the water-soluble phosphorus method using ammonium paramolybdate and ascorbic acid as described by Olsen and Sommers (1982). Control flasks were not inoculated, and had a pH of 7.20 and a water-soluble P content of 1.8 mg L⁻¹ after autoclaving. After 360 h incubation the control flasks had a pH of 6.06 and contained 7.5 mg L⁻¹ soluble P. Values obtained with the uninoculated controls were always subtracted from their respective treatments. All experiments were performed in triplicates.

Statistical analysis

The experimental design used to analyse the P solubilization results obtained in solid and liquid media was a split plot in time based on completely randomized design (bacterial groups as main plot and time of measurements as subplot). Variance homogeneity determination (ANOVA) was conducted with the General Linear Models of SAS by using the type II sum of squares, and means were compared according to the Duncan test (SAS, 1990).

Results and discussion

Preliminary assays with culture media

In preliminary studies, we modified the well-known rhizobia YMA medium (Vincent, 1970) by replacing the soluble source of P (K₂HPO₄) with 2.5 g L⁻¹ TCP or IHP and by adding 0.1 g L⁻¹ of KCl as a source of K. On modified media, no clear P solubilization halos were observed in solid media, and P release in liquid media from TCP was negligible. These results indicated that mannitol was not a good C source for P mobilization studies in rhizobia, and therefore all tests were performed with Sperber medium (1958) containing glucose as C source.

Phosphate solubilization in solid media was greatly affected by the C source used, and generally the larger calcium phosphate solubilization halos were obtained with glucose (Silva Filho and Vidor, 2000).

P mobilization in the solid Sperber medium

From the 446 strains of the Iranian rhizobia used in this study, 198 (44%) and 341 (76%) were able to mobilize TCP and IHP respectively. Antoun et al. (1998), tested 266 strains obtained from different laboratories in Australia, Columbia, Egypt and North America on the solid Goldstein (1986) medium supplemented with vitamins (Vincent, 1970), and found that 144 (54%) were dicalcium phosphate (DCP) solubilizers. The differences observed can be explained by the different calcium phosphate and nitrogen sources used. In the present work, yeast extract was used as nitrogen and vitamin sources while Antoun et al. (1998) used NH₄Cl as a nitrogen source. In developing efficient growth medium for screening PSM, yeast extract was avoided because of its inhibitory effect at concentration higher than 0.5 g L⁻¹ (Nautiyal, 1999). However, Halder and Chakrabarty (1993) also observed that the inorganic P solubilization activity of some *Rhizobium* strains was better in a medium without NH₄⁺, containing 0.4 g L⁻¹ of yeast extract as the nitrogen source. Rhizobia have different vitamin requirements (Vincent, 1970) that are better satisfied by yeast extracts. In some studies, the plate screening method has produced contradictory results between plate halo detection and P solubilization in liquid cultures. However this method can be regarded as generally reliable for isolation and preliminary characterization of PSM (Rodriguez and Fraga, 1999). In our study the plate method was very practical for screening a very large number of rhizobial isolates, however the procedure developed by Gupta et al. (1994) using bromophenol blue to improve detection of acid production and its adaptation to liquid media (Mehata and Nautiyal, 2001) should be further evaluated in future screening work.

None of the 57 strains of *Bradyrhizobium japonicum* and of the 13 strains of *Bradyrhizobium* sp. tested were able to mobilize P from TCP in Sperber solid or liquid medium. This observation suggests that *B. japonicum* strains are not

good inorganic P-solubilizers. In fact, Antoun et al. (1998) reported that only 1 out of the 18 strains of *B. japonicum* tested was able to mobilize P from DCP on a solid medium. The analysis of variance indicated that on the solid Sperber medium, the different groups of rhizobia mobilized P from TCP or IHP in a different manner (Table 1). Within each group a significant ($P < 0.001$) strain effect was also observed, indicating that the activity of the strains may vary significantly. Overall, strains of *Rhizobium leguminosarum* bv. *viciae* mobilized significantly ($P < 0.05$) more P from TCP than strains of *Mesorhizobium*, *Sinorhizobium* and *R. leguminosarum* bv. *phaseoli* (Table 2). The solubilization activity of the groups exhibited different trends at different time, as indicated by the significant ($P < 0.001$) group \times time interactions observed (Table 1). However, in general for all strains tested, the solubilization activity of TCP and IHP by the strains significantly ($P < 0.05$) increased with time (results not shown).

Soils may contain a substantial quantity of organic P (Richardson 2001), and phosphatases from microorganisms may carry out mineralization of most organic phosphorus compounds. From 30 up to 63% of culturable soil bacteria can mineralize organic P in soils (Rodriguez and Fraga, 1999). More rhizobia were able to mobilize P from IHP than from TCP. In fact 341 (76%) of the 446 Iranian rhizobia were able to mineralize IHP. With the exception of the *Bradyrhizobium* group (5–7%), 70% or more of the other rhizobial isolates were able to mineralize IHP. The only strain of *Bradyrhizobium* spp. able to mobilize IHP, had the highest observed mineralization halo/colony ratio. This solubilization activity was comparable to that observed with the strains of the *M. ciceri* and *M. mediterraneum* group, and was significantly ($P < 0.05$) higher than that of the other groups tested. As observed with TCP, the strain effect within each group on IHP solubilization is very significant ($P < 0.001$).

Table 1. ANOVA of the solubilization of inorganic ($\text{Ca}_3(\text{PO}_4)_2$) and organic (inositol hexaphosphate) phosphorus on the solid Sperber medium by rhizobia isolated from Iranian soils

Source of variation	Inorganic P		Organic P	
	Degree of freedom	Mean square	Degree of freedom	Mean square
Rhizobial group (G)	3	56.16***	6	404.33***
Strains	194	4.81***	333	9.61***
Time (T)	2	37.89***	2	270.52***
Interaction G \times T	6	12.43***	12	51.19***
Error	2170	0.06	3726	0.12

***Significant at $P < 0.001$.

Table 2. Solubilization of inorganic ($\text{Ca}_3(\text{PO}_4)_2$) and organic (inositol hexaphosphate) phosphorus on the solid Sperber medium by rhizobia isolated from Iranian soils.

Inorganic P		Organic P	
Rhizobial group	DH/CD	Rhizobial group	DH/CD
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	2.48a	<i>Bradyrhizobium</i> spp.	4.68a
<i>Mesorhizobium ciceri</i> & <i>M. mediterraneum</i>	1.42b	<i>Mesorhizobium ciceri</i> & <i>M. mediterraneum</i>	3.65a
<i>Sinorhizobium meliloti</i>	1.40b	<i>Sinorhizobium meliloti</i>	2.29b
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	0.96b	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	2.06bc
		<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	1.39bc
		<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	1.19bc
		<i>Bradyrhizobium japonicum</i>	0.83c

Values are the ratio of dissolution halo (DH)/colony diameter (CD).

Results are mean of three replicates, and three measurements made after 10, 20 and 30 days of incubation. Means followed by the same letter are not significantly different at $P < 0.05$.

P mobilization in the liquid Sperber medium

Strains producing halo/colony ratios higher than 1.2 on TCP plates (3, *Rhizobium leguminosarum* bv. *phaseoli*; 12, *R. leguminosarum* bv. *viciae*, 69, *Sinorhizobium meliloti*; and 64 *Mesorhizobium ciceri* & *M. mediterraneum*) were further investigated in liquid medium. All strains tested solubilized some P from DCP and produced acid in liquid culture. As observed on the solid medium, bacterial groups and strains within each group had significantly ($P < 0.001$) different solubilization and acid production activities (Table 3). The soluble P released by the strains significantly ($P < 0.05$) increased with time (Figure 1).

Table 3. ANOVA of phosphorus mobilized from $\text{Ca}_3(\text{PO}_4)_2$ and of the change in pH of the liquid Sperber medium inoculated with rhizobia isolated from Iranian soils and with phosphate-solubilizing bacteria (one isolate of *Bacillus* sp. and three isolates of *Pseudomonas fluorescens*) used as positive controls

Source of variation	Degree of freedom	Mean squares	
		Phosphorus	PH
Bacterial group (G)	4	557658.20***	21.64***
Strains	147	35085.25***	2.31***
Time (T)	3	2478319.23***	33.18***
Interaction G \times T	12	25294.82***	1.51***
Error	1657	1492.10	0.1

***Significant at $P < 0.001$.

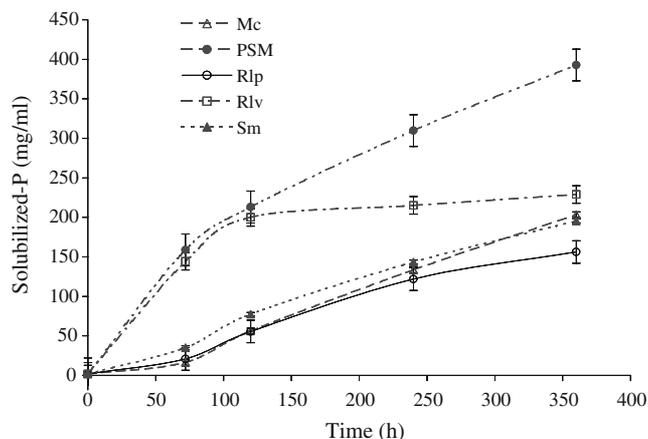


Figure 1. Solubilization of $\text{Ca}_3(\text{PO}_4)_2$ in the liquid Sperber medium by bacterial isolates belonging to the following groups: Mc, *Mesorhizobium ciceri* and *M. mediterraneum*; PSM, phosphate-solubilizing *Bacillus* sp. and *Pseudomonas fluorescens* used as controls; Rlp, *Rhizobium leguminosarum* bv. *phaseoli*; Rlv, *R. leguminosarum* bv. *viciae*; Sm, *Sinorhizobium meliloti*. Error bars are \pm standard error ($n = 3$).

The four isolates of PSM (*Bacillus* sp. and *Pseudomonas fluorescens*) used in this study as positive controls released an average of 268.6 mg mL^{-1} of P from TCP. This quantity was significantly ($P < 0.05$) higher than the 197.1 mg mL^{-1} of P mineralized by strains of the group *R. leguminosarum* bv. *viciae*. The other three groups of rhizobia released the following comparable amounts of soluble P which are significantly lower ($P < 0.05$) than those obtained with PSM and *R. leguminosarum* bv. *viciae*: *S. meliloti*, 112.8 mg mL^{-1} ; *M. ciceri* and *M. mediterraneum*, 102.3 mg mL^{-1} ; and *R. leguminosarum* bv. *phaseoli*, 88.66 mg mL^{-1} . As revealed by statistical analyses, the results obtained in the TCP liquid medium corroborate those observed with the solid medium, indicating that strains of the group *R. leguminosarum* bv. *viciae* isolated from Iranian soils are the more effective TCP solubilizers. Halder and Chakrabarty (1993) previously reported that strains of *R. leguminosarum* bv. *viciae* can achieve high inorganic P solubilization.

Significant drops in pH accompanied the release of soluble P from TCP, in the culture supernatants (Table 3 and Figure 2). This confirms the implication of organic acid production in P solubilization by rhizobia (Halder and Chakrabarty, 1993). For all groups of rhizobia tested, strong significant ($P \leq 0.01$) inverse correlations ($r = -0.66$ to -0.89) were observed between the pH of the culture supernatants and

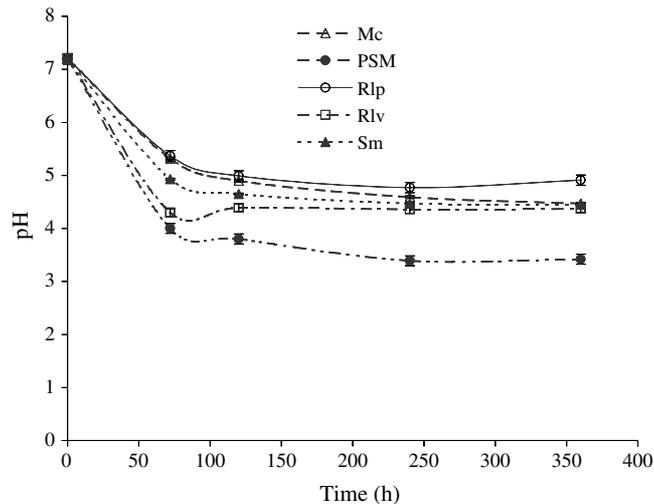


Figure 2. Changes of the pH of the culture filtrates of the liquid Sperber medium during the solubilization of $\text{Ca}_3(\text{PO}_4)_2$ by the different bacterial groups tested. For abbreviations see Figure 1. Error bars are \pm standard error ($n = 3$), and are smaller than the symbols.

their soluble P content, corroborating similar observations made with rhizobia (Halder and Chakrabarty, 1993), and other bacteria mobilizing P from rock phosphate (Nahas, 1996).

These results indicate that many rhizobia are able to mobilize P from inorganic and organic sources. These rhizobia also have proved to be good plant growth PGPR with non-legumes (Antoun et al., 1998; Yanni et al., 2001). In developing inoculants that improve plant P nutrition and allow plants to use soil stocks of organic and inorganic P, rhizobia may present many advantages. In fact, in addition to their beneficial effects on legume and non-legume plants which will be an advantage in crop rotation systems, inoculation and inoculants production technologies are already available, and rhizobia are generally perceived as environmentally friendly, since they have been used with legumes for many years without causing harm to the environment or to farmers (Antoun et al., 1998).

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