Soil & Environ. 27(1): 71-76, 2008

Efficiency of three *Pseudomonas* isolates in releasing phosphate from an artificial variable-charge mineral (iron III hydroxide)

A. Ghaderi[1], N. Aliasgharzad[1*], S. Oustan[1] and P.A. Olsson[2]
[1]Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, I.R. of Iran
[2]Plant Ecology and Systematics, Department of Ecology, Lund University, Lund, Sweden

Abstract

Phosphorus management in agriculture involves acquiring exact and comprehensive information about the fate of added phosphate to the soil. A great part of a phosphate (P) fertiliser is retained on the surface of variable-charge minerals through specific adsorption; the precipitation process takes place in the limited space around the fertiliser grains where its concentration is high. We tested the ability of three Pseudomonas strains to release P from iron (III) hydroxide. Iron hydroxide precipitated on filter paper, was used as a variable-charge mineral and P was loaded on it. The siderophore and H^+ production, acid and alkaline phosphatases activity were assayed in vitro. All three isolates released P as compared to controls with killed bacteria or without bacteria. The amount of released P from iron hydroxide were 50.5, 29.2 and 61.6% for P. putida, P. fluorescens Chao and P. fluorescens Tabriz, respectively. The amount of P in solution phase was highest (0.74 mg P/50mL) with P. putida and lowest (0.32 mg P/50 mL) with P. fluorescens Tabriz. The highest P assimilation from total P released was in P. fluorescens Tabriz. In vitro tests revealed that the P. fluorescens Chao with significantly higher siderophore production and phosphatases activity, had the least effect on P release from iron hydroxide, while two other isolates had significantly higher H^+ production. It seems that the H^+ production is the main mechanism of P released from variable charge minerals and P. putida was the most efficient bacterium.

Key words: *Pseudomonas,* phosphate releasing, variable-charge minerals, specific P adsorption

Introduction

A large fraction of phosphate fertilizers added to soil is rendered immobile, which reduce its availability for plants and other organisms. In soils with high lime content and pH, it seems as the major part of added P is precipitated as Ca-phosphate. It has been shown that a great part of phosphate is retained on the surface of variable-charge minerals (VCM) (Barrow, 1980) by specific adsorption, and the precipitation process takes place only in the limited space around fertilizer grains where the P concentration is high (He and Zhu, 1997, 1998). Chemical fixation of P in VCM soils is generally attributed to the adsorption of phosphate on the surface of Fe and Al oxides. This adsorption leads to low efficiency of phosphate fertilizers. The goethite-P complexes have the lowest release rate among P complexes. This may imply that iron oxides are the main part of VCM in soils (Barrow, 1987; He and Zhu, 1997). It has been shown that the added P adsorbs on the surface of Fe and Al oxides and then slowly transforms into other forms (Cornforth, 2005; Chen *et al*., 2006).

Some soil microorganisms dissolve inorganic and organic phosphates that have low solubility (Rodriguez and Fraga, 1999). He and Zhu (1997) showed that the $NaHCO_3$-extractable phosphate (Olsen *et al*., 1954) is increased in the presence of phosphate solubilizing microorganisms (PSM). They demonstrated that the sum of $NaHCO_3$-extractable P and microbial biomass P was 70-80% of the total P added to the soil. Certain *pseudomonads* are efficient in solubilizing rock phosphate, as has been shown for *Pseudomonas putida* (Kumar and Singh, 2001; Villegas and Fortin, 2002; Pandy *et al*., 2006; Rosas *et al*., 2006), *P. fluorescens* (Alikhani *et al*., 2006), *P. striata* (Kucey, 1983; Premono *et al*., 1996; Kumar and Singh, 2001), *P. jesseni* (Valverde *et al*., 2006) and *P. aeroginosa* (Villegas and Fortin, 2002).

Acid (H^+) production is one of the most important mechanisms in dissolving mineral phosphate (Igual *et al*., 2001; Richardson, 2001). Several researchers pointed out that the release of low molecular organic acids is a possible response of microbes to low availability of phosphorus (Goldston, 1986; Kim *et al*., 1997; Hilda and Fraga, 1999). Chen *et al*. (2006) reported that PSM produced organic acids such as citric, oxalic, gluconic, lactic, succenic and propionic acids. Among them, citric acid gave maximum pH decrease and P solubilization. Ligands in organic acids may also have considerable effect on mineral phosphate solubilization.

Siderophore production is another mechanism for dissolution of low soluble phosphate. Siderophores are low molecular weight compounds synthesized under

*Email: n-aliasghar@tabrizu.ac.ir

iron-deficient conditions by many of microorganisms. They chelate Fe^{+} and facilitate its transport into the cell through receptor mediation (Beckie *et al*., 1998). Siderophore production by *Pseudomonas* species has been reported for *P. fluorescens* (Meyer and Abdallah, 1978; Philson and Llinas, 1982; Baysse *et al*., 2002), *P. putida* (Boopthi and Rao, 1999; Pandy *et al*., 2006) and *P. aeruginosa* (Villegas and Fortin, 2002).

Iron hydroxide-impregnated filter papers have been used to study the availability of phosphorus to plants (Menon *et al*., 1988). The papers are prepared by immersing them in $FeCl_3$ and NH_4OH solutions, respectively. In this way, an artificial Fe(III)-hydroxide, similar to VCM, is achieved. Some modifications to this method have been proposed by Lin *et al.* (1991) and Bramley and Roe (1993).

We isolated five *Pseudomonas* strains that showed the ability to release P from Ca-phosphate. These isolates were tested for their ability to increase plant P uptake from mineral sources. Of these, three isolates were the most efficient in P solubilization (Rasipour and Aliasgharzad, 2006). In this study, these three isolates were tested *in vitro* for their ability to release P also from Fe hydroxide. We tested the hypothesis that P releasing ability was due to H^{+} and siderophore production and we investigated the effect of phosphatase production on P fractionation *in vitro*.

Materials and Methods

Biological material

Psedomonas fluorescens Chao was obtained from Plant Pathology Research Institute of Tehran, Iran. This strain is usually used as a biocontrol agent against fungal root diseases due to production of siderophore and antibiotics (Meyer and Abdallah, 1978; Philson and Llinas, 1982). *Psedomonas putida* and *Psedomonas fluorescens* Tabriz were isolated from soils of Agricultural Research Station of Tabriz University, Iran, as described by Moore *et al*. (2006). In our previous study, all three strains were shown high potential to solubilize tri-calcium phosphate in Sperber's medium (Rasipour and Aliasgharzad, 2006).

Experiment (1)

Preparation of iron hydroxide-impregnated filter paper

First, Whatman hardened filter papers No. 542 (11 cm, diameter; 2.7 µm, pore size) were immersed in 10% $FeCl_3$ solution for 30 min. Then they were dried for one hour in room temperature. After that, they were rapidly dipped in a 5% ammonium hydroxide solution and washed three times in distilled water (Lin *et al*., 1991).

Phosphate loading on iron hydroxide

The filter papers were folded and placed in 100 mL flasks. In order to prevent adhesion of the filter paper to the flask wall, they were fastened by nylon bands in a way that solution could pass through it. Fifty ml of KH_2PO_4 solution (5 g P L^{-1}) was added to each flask and were shaken (140 rpm) for an equilibration time of one week at room temperature. Then fliter papers were rinsed three times with distilled water. We have earlier found that the 5 g P L^{-1} is a suitable concentration for maximum P loading on iron oxide-impregnated filter paper (11 cm diameter) as specific adsorption with no culmination of precipitation according to the method described by Sharpley (1993) and this was equal to 13 mg P/filter paper.

Culture medium and treatments

Culture medium for bacteria was P free King´s B, buffered at a pH of 7.2. Fifty ml of medium and one Fe hydroxide-filter paper were added to each 100 ml flask and autoclaved (120 ºC, 1 atm, 30 min). Three *Pseudomonas* isolates, *P. putida* Tabriz, *P. fluorescens* Tabriz and *P. fluorescens* Chao, were grown in LB medium (Alef and Nannipieri, 1995) for two days and their population densities were adjusted to $5x10^{7}$ cell mL^{-1} according to OD_{600} measurements. Each flask was inoculated with one ml of respective bacterial suspension and maintained on a horizontal shaker-incubator for two weeks at 26ºC and 140 rpm. Two controls were applied in this experiment. Control I had the same composition as treatments (with *P. fluorescens* Tabriz) but one ml chloroform was added before the experiment in order to kill the bacteria. P measurement in control I was done immediately after chloroform addition. This control indicates P content in the bacterial suspension. Control II consisted of 50 mL King´s B medium plus filter paper, thus indicating the amount of chemically P desorption from iron hydroxide by the medium. All treatments and controls had four replications.

Measurement of P fractions

After two weeks of experimental treatment, filter papers were removed and the suspensions were transferred to centrifuge tubes and centrifuged at 16000×g for 20 min. The supernatants were analysed for inorganic P, and then digested with a mixture of H_2SO_4 and HNO_3 (Rand *et al*., 1997) for determining total P. Organic P in the supernatant was calculated by subtracting inorganic P from total P. Phosphorus content

of the bacterial pellet was determined after digestion as described above. All P measurements were carried out using the ascorbic acid-molybdenum method (Olsen and Sommers, 1982).

Experiment (2)

In vitro assays

In vitro tests included H^+ production in King´s B containing bromochresol green (Alef and Nannipieri, 1995), siderophore production in King´s B medium containing chrome azurol-S (Bernhard and Neilands, 1987), and acid and alkaline phosphatases in P free King´s B medium containing Sodium-paranitrophenyl phosphate as substrate (Alef and Nannipieri, 1995). These tests were performed on each bacterial isolate with three replications.

Statistical analysis

Analysis of variance (1-way ANOVA) and Douncan´s Multiple Range Test for means were carried out using SAS software v.7.

Results and Discussion

All three bacterial isolates increased significantly the P release from the iron hydroxide substrate. The amount of total P released in Type I controls was 0.018 mg (n=4) and from type II controls 0.68 mg (n=4).

All P fractions were significantly ($p<0.001$) influenced by the type of bacterial isolate Total P released was highest with *P. fluorescens* Tabriz and lowest with *P. fluorescens* Chao (Figure 1). Supernatant analysis showed that the inorganic P was considerably higher in *P. putida* treatment than *P. fluorescens* Tabriz (Figure 2). In contrast, the highest organic P in the supernatant was produced by *P. fluorescens* Tabriz while *P. fluorescens* Chao produced least (Figure 3).

Inorganic P in solution phase is readily available for plants, indicating that *P. putida* could be efficient in supplying P for plants from VCM surfaces, although, organic P produced by both *P. fluorescens* Tabriz and *P. putida* could be available for plants when subjected to phosphatase activity.

Phosphorus assimilation by the bacteria was significantly ($p<0.05$) different between isolates. *P. fluorescens* Tabriz assimilated the highest amount of P while the minimum was found in *P. putida* (Figure 4). There was a negative relationship between inorganic P in supernatant (solution phase) and P assimilation (biomass P). The ratio of total P in supernatant to biomass P (TPS/BP) could be a useful parameter for explanation of bacterial efficiency in supplying P to the plants. Considering this criterium, *P. putida* had the highest ratio (Table 1). A high ratio indicate high availability of P, and it means that the bacterial demand for P is low.

The percentage of total P in supernatant from total P released (TPS/TPR)×100 is another parameter to asses the ability of bacteria in releasing P to solution phase which in turn reflects the amount of P assimilation. This ratio was 67% for *P. putida,* 50% for *P. fluorescens* Tabriz and 23% for *P. fluorescens* Chao (Table 1). He and Zhu (1998) found that certain soil microorganisms release 36-46% of P adsorbed on VCM during 21 days of incubation. Although most of the P in the supernatant is in the organic form, it could be available for plants on a short time basis in soil systems due to phosphatase activity. Many soil microorganisms produce phosphatases in response to P deficiency (Kucey, 1983; Alef and Nannipieri, 1995).

In vitro experiments revealed that both *P. putida* and *P. fluorescens* Tabriz have significantly higher H^+ production (pH decline) than *P. fluorescens* Chao (Table 2). Considering the higher P release in two former bacteria, it seems that the H^+ production is the main mechanism in releasing P from VCM. There are two mechanisms by which inorganic P might be released from VCM surfaces: (i) ligand exchange, and (ii) H^+-promoted dissolution of VCM. The extent of ligand exchange is pH dependent. Since the release of P is coupled with an incorporation of OH^- ions onto VCM surfaces, release is favoured by higher pH values. This is not the case here. In the second process, dissolution of the Fe-oxide surface releases adsorbed P in response to decreasing pH. It can be concluded that the latter processe is the case in our study. Solubilization of rock phosphate by certain pseudomonads as a result of pH decline in their medium, has been reported by Chen *et al*. (2006), Babana and Antoun (2006) and Rosas *et al.* (2006).

P. fluorescens Chao had significantly higher phosphatase activity and siderophore production in comparison with the two other isolates in *in-vitro* assessment (Table 2). Based on our results, siderophore as a ligand had little or no effect on releasing phosphate from VCM by ligand exchange mechanism, because the lowest phosphate concentration in solution phase was found in the presence of this bacterium (Experiment 1). It seems that the P bounded to $Fe(OH)_3$ is not reachable by siderophores. Phosphatases only act on organic-bound phosphate and had no effect on releasing P from mineral complex. Therefore, *P. fluorescens* Chao can play a key role in phosphate solubilization in organic soils due to their higher content of organic phosphorus.

Table 1. Percentage of total P in supernatant and proportion of P assimilation by the bacterial isolates

	P. putida	*P. fluorescens* Chao	*P. fluorescens* Tabriz
(TPS/TPR)×100	67% a*	23% c	50% b
TPS/BP	1.89 a	0.33 c	1.10 b

TPS, total P in supernatant; TPR, total P released; BP, biomass P
*Means in each row followed by same letter are not significantly different (p< 0.05).

Table 2. Production of H^+, siderophore, acid and alkaline phosphatases by the bacterial isolates (Data are ratio of halo to colony diameter)

Isolates	H^+ production	Siderophore	Acid phosphatase	Alkaline phosphatase
P. putida	2.2 a*	2.0 b	1.1 b	1.1 b
P. fluorescens Chao	1.7 b	2.4 a	2.2 a	2.0 a
P. fluorescens Tabriz	2.3 a	2.1 b	1.0 b	1.0 b

*Means in each column followed by same letter are not significantly different (p< 0.05).

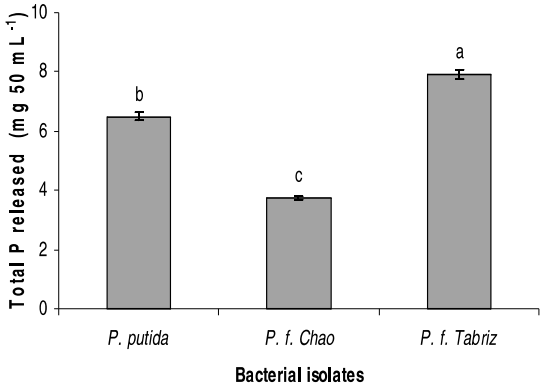

Figure 1. Effect of bacterial isolates on total P released from VCM (*P.f*, *Pseudomonas fluorescens*). One-way ANOVA (p< 0.001).Different letters indicate significant differences between means (p < 0.05)

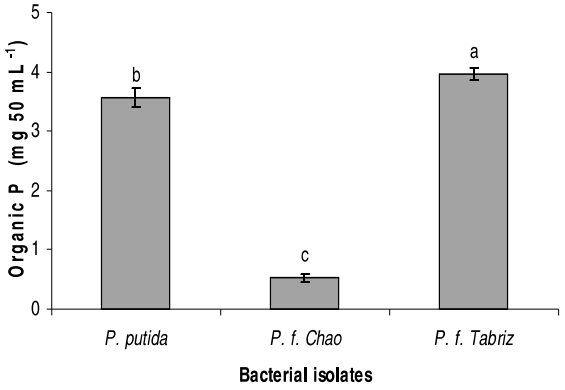

Figure 3. Effect of bacterial isolates on organic P in supernatant (*P.f*, *Pseudomonas fluorescens*). One-way ANOVA (p< 0.001).Different letters indicate significant differences between means (p < 0.05)

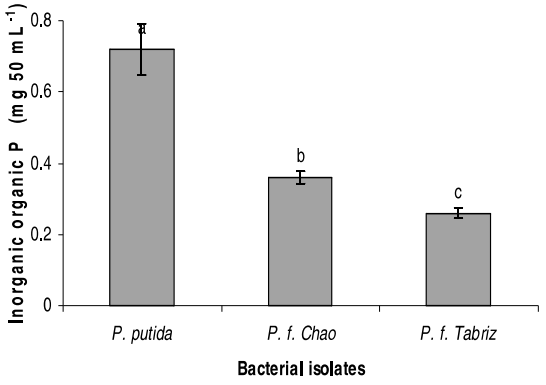

Figure 2. Effect of bacterial isolates on inorganic P in supernatant (*P.f*, *Pseudomonas fluorescens*). One-way ANOVA (p< 0.001). Different letters indicate significant differences between means (p < 0.05)

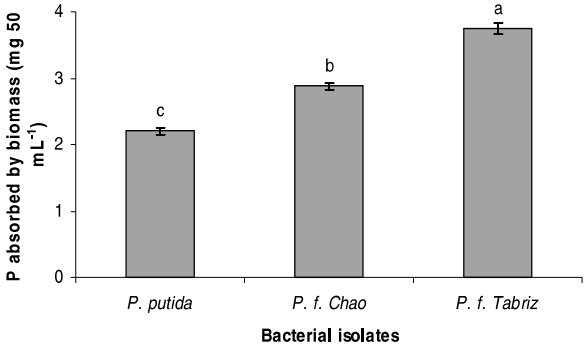

Figure 4. Amount of P assimilation from total P released by the bacterial isolates (*P.f*, *Pseudomonas fluorescens*). One-way ANOVA (p< 0.001). Different letters indicate significant differences between means (p < 0.05)

Conclusion

Phosphate tightly bound to VCM is not available to plants, but some soil microorganisms have ability to release it. Based on our results, all three bacterial isolates used in this study were able to release P from Fe(III) hydroxide. Among them the *P. putida* was the most efficient because it released P (67% of total released) into solution phase, which is completely available for plants. *P. fluorescens* Tabriz also had high efficiency in desorbing P from iron hydroxide but only 50% of total P was released to solution phase and the remaining was assimilated in bacterial cells. Total P released by *P. fluorescens* Chao was the least and therefore the relatively lower P was in the solution phase.

Among mechanisms involved in P releasing from VCM by the bacteria which were tested in this study, H^+ production was the most efficient. Acidification of medium by the *P. putida* has already been reported (Premono *et al.,* 1996; Pandy *et al*., 2006; Rosas *et al*., 2006). H^+ production enhances dissolution of VCM which leads to release of phosphate ions into solution phase.

P. putida used in this study could be an efficient bacterium as phosphate biofertilizer in agricultural soils. Also, *P. fluorescens* Chao could be an efficient phosphate-releasing bacterium in soils with high content of organic matter, due to its higher potential for producing acid- and alkaline phosphatases. However, recommendation of these bacteria in a broader sense needs further investigations, especially under field conditions.

Acknowledgment

The financial support provided by the Faculty of Agriculture, University of Tabriz is gratefully acknowledged.

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