



## Solubilization of Inorganic Phosphate by Endophytic *Pseudomonas* sp. from French Bean Nodules

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**Summary:** Phosphorus (P) is one of the major essential macronutrient for plant growth. Phosphate solubilizing bacteria (PCB) is a group of heterotrophic bacteria capable of solubilizing organic and inorganic phosphorus from insoluble compounds, releasing phosphate available for plant uptake. In this study endophytic bacteria were isolated from nodules of French bean grown in different intercropping systems. Only 14 out of 158 endophytic isolates were able to solubilize tricalcium phosphate (TCP). None of them were observed to solubilize  $\text{FePO}_4$  and  $\text{AlPO}_4$ . Eight isolates formed solubilization zones larger than 3 mm and were used for further test. The TCP solubilization was measured in liquid Pikovskaya medium and ranged from 14-182  $\mu\text{g mL}^{-1}$  of soluble phosphorus. TCP solubilization was correlated with acidity and pH decreasing from 7.4 to 5.57 and 4.91 for the lowest and the highest solubilizer, respectively, were observed. The most efficient isolate MP18 was selected and identified as *Pseudomonas* sp. according to 16S rRNA gene analysis.

**Keywords:** endophytic bacteria, French bean nodules, phosphate solubilizing bacteria, *Pseudomonas*, soil pH

### Introduction

Phosphorus (P) is an essential mineral nutrient most commonly limiting the growth of crops. The phosphate fertilization of soils has always been important because vast areas of agricultural land can be poor if the soil has a phosphate deficiency. Phosphorus is typically insoluble or poorly soluble in soils (Banerjee et al. 2010). Soil P dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes (Khan et al. 2009). Anthropogenic addition to the soil can easily be precipitated into insoluble forms such as TCP-tricalcium phosphate [ $\text{Ca}_3(\text{PO}_4)_2$ ],  $\text{FePO}_4$  and

$\text{AlPO}_4$  (Banerjee et al. 2010). Anthropogenic addition of P fertilizers can often have negative effects on ecosystems such as toxic effect on plants and freshwater eutrophication (Madigan & Martinko 2006). For these reasons, one very attractive approach for insoluble forms of phosphorus is the application of microorganisms. This group of bacteria can be found in the rhizosphere, at root surface and in association with roots.

Presence of plant growth promotion rhizobacteria (PGPR) can improve extent or quality of plant growth directly or indirectly (Mehta et al. 2010). PGPR are directly involved in solubilization of minerals such as phosphorus; stimulation of seedlings root length increased uptake of nitrogen; production of siderophores that chelate iron and

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make it available to the plant root; synthesis of vitamins and phytohormones: indoleacetic acid (IAA), gibberellic acid, cytokinins, ethylene and efficient uptake of water (Meunchang et al. 2006, Mehta et al. 2010, Yasmin et al. 2012). Indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effect of many pathogens, insects and nematodes through the production of antibiotics, lytic enzyme and hydrogen cyanide (Chaiahrn & Lumyion 2008, Yasmin et al. 2012).

The phosphate-solubilizing microorganisms (PSM) are capable of hydrolysing organic and inorganic phosphorus from insoluble compounds, releasing phosphate available for plant uptake (Ahmad et al. 2008, Chaiahrn & Lumyion 2008). Compared to fungi, phosphate solubilizing bacteria (PSB) are more effective in phosphate solubilization, which causes the wider potential uses of bacteria – they play a significant role in making phosphorus available to plants and enable the usage of less available sources of plant nutrients, such as rock phosphate. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acid synthesized by soil microorganisms. Production of organic acid results in acidification of the microbial cell and its surroundings (Khan et al. 2009).

The aim of this study was to select endophytic bacteria from nodules of French bean grown in different intercropping systems with high ability of phosphate solubilization.

## Materials and Methods

French bean was grown in agricultural field of Institute for Vegetable Crops, Smederevska Palanka (44°21'N, 20°57'E), central Serbia. Endophytic bacteria were collected from the nodules of French bean grown in different systems: sole crop and intercropped with red beet and radish. Nodules formation was induced by indigenous rhizobia in all intercropping systems. Nodules were sterilized and bacteria were isolated on Yeast Manitol Agar (YMA) (Jošić et al. 2008). Pure cultures were obtained on YMA, King's B agar (KB) and Nutrient agar (NA) by restreaking several times (Djuric et al. 2011).

All bacterial isolates were screened on Pikovskaya (PVK) ( $\text{gl}^{-1}$ : yeast extract, 0.5; agar, 15; glucose, 10;  $\text{Fe SO}_4$ , 0.0001;  $\text{MnSO}_4$ , 0.0001;  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.1; KCl, 0.2;  $(\text{NH}_4)_2\text{SO}_4$ , 0.5) growth medium supplemented with  $5\text{gl}^{-1}$  TCP for P-solubilization potential. Bacterial colonies surrounded by a halo (indicating removal of phosphate) on PVK medium were assumed to be capable for P-solubilization and selected for further assay on PVK and National Botanical Research Institutes Phosphate (NBRIP) ( $\text{gl}^{-1}$ : glucose, 10;  $(\text{NH}_4)_2\text{SO}_4$ , 0.1; KCl, 0.2;  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.25;  $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ , 5; agar, 15) mediums (Kapri & Tewari 2010). Insoluble TCP,  $\text{FePO}_4$  and  $\text{AlPO}_4$  in concentration of 4–6  $\text{gl}^{-1}$  were used as sources of phosphate in both mediums. The plates were prepared 24 h earlier and  $10\mu\text{l}$  of  $5 \times 10^8 \text{ CFUml}^{-1}$  bacterial suspensions were injected in medium in triplicate. The plates were incubated

Table 1. Isolates from French bean nodules able to solubilize TCP and control strains used in this study

Isolate	Locality	French bean intercropped with:
MP2	SP- S11	radish
MP3	SP- S11	radish
MP7	SP- K11	radish
MP8	SP- N1	/
MP9	SP- N7	red beet
MP17	SP- K1	/
MP18	SP- K11	radish
MP19	SP- N7	red beet
Control strains		
k-: <i>Bacillus</i> sp. strain B2	Pančevo	soil
k+: <i>Pseudomonas</i> sp. strain Q4	Arandelovac	maize rhizosphere

at 26°C and halo zones were measured at 7 days of incubation.

Only isolates showing P-solubilization zone of 3 mm and more were selected for quantitative analysis (Table 1). Quantitative estimation of solubilized P was carried out in 25 ml of Pikovskaya broth amended with 5g/l TCP. Before inoculation, the bacteria were cultured on Nutrient Broth at 26°C for 24h and adjusted to  $5 \times 10^8$  CFUml<sup>-1</sup>. Medium was inoculated with  $5 \times 10^6$  CFU of each isolate, incubated at 27°C for 4 days at 150 rpm and cultures were harvested by centrifugation at 5400 rpm for 10 min. Total P amount in supernatants were estimated spectrophotometrically (Jelenić & Džamić 1985).

Taxonomic characterization of selected isolate MP18 was performed on the basis of Gram staining, biochemical properties using API 20 NE test (Biomerieux, France) and genetic characterization of 16S rRNA gene. Total DNA were isolated and 16S rDNA amplicon were yielded using fD1/rD1 primer set (Weisburg et al. 1991, Jošić et al. 2012a). Sequence of obtained amplicon was compared to those in NCBI GenBank.

## Results and Discussion

Total of 42.4% (67 out of 158) of endophytic bacteria isolated from French bean nodules

belongs to indigenous rhizobia. All of 6 rhizobia that formed halo zones on PVK medium were concerned as poor TCP solubilizers, showing zones between 1 and 2 mm. Other bacterial genera were represented by 57.6% of isolates (91) and only 8.79% among them showed ability to solubilize TCP in PVK medium. Two out of 8 selected isolates were isolated from nodules of French bean grown as a sole crop and two from nodules of French bean intercropped with red beet, as shown in Table 1. Four isolates originated from nodules of French bean near radish rhizosphere.

No growth was observed on NBRIP supplemented with FePO<sub>4</sub> and AlPO<sub>4</sub> and PVK supplemented with AlPO<sub>4</sub>. Control strains and 3 isolates grew on PVK supplemented with FePO<sub>4</sub>, but no halos were observed around the colonies. Since the direct measurement of phosphate solubilization in broth assay is likely to give more reliable results than a regular plate assay, the eight phosphate-solubilizing strains were further tested for their ability to solubilize TCP in PVK broth. The values of P-solubilization zones, pH values and concentration of solubilized TCP in liquid PVK medium are shown in Table 2.

The selected isolates showed a wide range of TCP solubilization after four days: 14-182 µgml<sup>-1</sup>. MP18, isolated from nodules of French bean intercropped with radish, showed the highest phosphate

Table 2. Growth and phosphosolubilization ability on two solid (s) mediums and phosphate concentration in Pikovskaya liquid (lq) medium

Isolate	Medium				
	PVK (s) FePO <sub>4</sub> <sup>a,b,c</sup>	PVK (s) Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> <sup>b</sup>	NBRIP (s) Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> <sup>b</sup>	PVK (lq) Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> <sup>b</sup>	
	growth/ P-solubilization	zone of P- solubilization (mm)	zone of P- solubilization (mm)	pH	conc. P (µg ml <sup>-1</sup> )
MP2	-	5	3.5	5.20	51
MP3	-	6	4	5.57	14
MP7	+/-	4.5	4	5.51	40
MP8	+/-	5	3.5	5.22	28
MP9	-	6	3	5.01	81
MP17	-	7	3.5	5.08	26
MP18	-	6	5.5	4.91	182
MP19	+/-	7	3.5	5.13	48
Q4	+/-	8	3	4.53	246
B2	+/-	4.5	4	5.40	29

\*concentration of insoluble phosphates added: <sup>a</sup> – 6 gl<sup>-1</sup>; <sup>b</sup> – 5 gl<sup>-1</sup>; <sup>c</sup> – 4 gl<sup>-1</sup>;

No growth: NBRIP supplemented with 4-6 gl<sup>-1</sup> FePO<sub>4</sub> and 4-6 gl<sup>-1</sup> AlPO<sub>4</sub> and PVK supplemented with 4-6 gl<sup>-1</sup> AlPO<sub>4</sub>

solubilizing efficiency among endophytic isolates ( $182 \mu\text{gml}^{-1}$ ) and reached 73.98% efficiency of rhizospheric *Pseudomonas* sp. Q4 strain used as positive control. The minimal value of TCP solubilization in liquid PVK medium was observed for isolate MP3 from the same intercropping system, despite the same value of P- solubilization zone in solid PVK as the most efficient isolate. TCP solubilization for MP3 was lesser than for *Bacillus* sp. B2 strain, originated from contaminated soil, used as poor TCP solubilizer. The value of  $81 \mu\text{g ml}^{-1}$  was observed for MP9 isolated from nodules of French bean intercropped with red beet. Similar results are reported for different rhizospheric *Pseudomonas* strains. The solubilization of TCP is reported for different rhizospheric fluorescent pseudomonads belonging to *P. monteilli*, *P. putida*, *P. plecoglossicida*, *P. fluorescens*, *P. fulva*, *P. monteilli* and *P. aeruginosa* and soluble phosphate ranged from 29 to  $105 \mu\text{gml}^{-1}$  after 10 days of inoculation (BMC) (Naik et al. 2008). The value of  $74.6 \mu\text{gml}^{-1}$  soluble phosphate from inorganic phosphate is observed for *P. fluorescens* strain NJ-101 isolated from agricultural soil (Bano & Mussarat 2004, Jošić et al. 2012a).

P-solubilization by MP18 was accompanied by a decrease in pH of the culture supernatants from 7.40 to 4.91. Similarly, the pH value of 4.53 was observed after 4 days of cultivation for *Pseudomonas* sp. Q4 strain. Similar pH decrease (to 4.8) was observed for numerous taxonomically different rhizospheric fluorescent pseudomonads (BMC) (Naik et al. 2008). Although phosphate solubilization is not necessarily correlated with acidity, a basic principle is related to the production of organic acids and the releasing of protons (Lin et al. 2006). It is correlated to our results for MP3 isolate where the lowest pH decreasing of medium (to 5.57) and the lowest TCP solubilization was recorded. The decrease in pH in relationship to release of soluble phosphates is also reported for free-living soil bacteria (Rajkumar & Freitas 2008) and rhizobia (Sridevi et al. 2007). The phosphate solubilization depends not only to the quantity of organic acid secreted, but to the structural complexity and particle size of phosphates and other mechanisms (Nautiyal et al. 2000).

Characterization of selected isolate MP18, as the most efficient, was based on biochemical characteristics obtained by API 20NE test and 16S rRNA gene sequence analysis. No fluorescent pigment production was observed on KB medium. Isolate MP18 was Gram-negative, motile, rod shaped, non-fluorescent, positive for cytochrome oxidase test and able to grow at  $4^{\circ}\text{C}$  and  $42^{\circ}\text{C}$ . Comparison with similar sequences in NCBI GenBank showed 99% similarity to several

*Pseudomonas* sp. and allowed to characterize it as *Pseudomonas* sp.

The bacteria belonging to *Bacillus*, *Achromobacter*, *Aerobacter*, *Acinetobacter*, *Enterobacter*, *Mesorhizobium*, *Rhizobium*, *Klebsiella* and *Micrococcus*, have been reported as phosphate solubilizers. However, strains belonging to *Pseudomonas*, especially fluorescent strains, are considered as efficient phosphate solubilizers (Villegas & Fortin 2002). Among endophytic bacteria isolated from root nodules in various leguminous plants (alfalfa, clover, soybean, pigeon pea) are *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Burkholderia* and *Enterobacteria* (Zakhia et al. 2006, Kan et al. 2007, Geetha et al. 2008, Li et al. 2008). The results from our study showed the most efficient phosphate solubilizing endophytic isolate MP18 belongs to *Pseudomonas* genera, according to phenotypic characters and 16S rRNA gene sequence. The plant growth promoting traits of different endophytic (Stajković et al. 2011) and rhizospheric *Pseudomonas* spp. from Serbia is already reported and most of them are able to solubilize TCP (Djuric et al. 2011, Josic et al. 2012b). Besides being vital for photosynthesis, energy transfer and formation of sugars, P also promotes nitrogen fixation in legumes (Gaur 1990). Low levels of phosphorus reduce the population of legume-nodulating microorganisms and affect rhizobia-legume symbiosis (Moreira et al. 2010). Phosphate solubilization occurred by rhizobia isolated from *Crotalaria* sp. (Sridevi et al. 2007). Only rhizobia able to solubilize TCP in very low rate were observed in our study. The natural simultaneous presence of rhizobia and *Pseudomonas* sp. MP18 in French bean nodule may be explained by low P-solubilizing capacity of indigenous rhizobia and possibility of MP18 to improve availability of phosphorus for microsymbiont and host-plant.

Numerous studies confirmed beneficial effects of legumes in dual inoculation with rhizobia and PSB. Rudresh et al. (2005) reported the increase in nodulation and biological nitrogen fixation due to synergistic effect of rhizobia and PCB comparing to their individual application. Significant response of dual inoculation was observed in chickpea crop, increasing yield and leghemoglobin content in the nodular tissues (Barea et al. 2005). The dual inoculation of endophytic strains *P. chlororaphis* Zong1 and *Mesorhizobium* sp. SQ1 cause synergistic or additive effect and improve not only phosphate solubilization, but also siderophores production, organic acid production, IAA production and *in vitro* antifungal activity, compared to single inoculation (Zhao et al. 2013).

## Conclusions

The selected endophytic *Pseudomonas* sp. MP18 isolate reached high phosphate solubilizing efficiency comparing to positive control. For the agricultural production it could be added to some microbial fertilizers and used especially on soils containing high amounts of insoluble TCP. Also, it could be suitable for organic production and other sustainable production systems. According to the obtained results, it will be further investigated for other PGP traits and synergistic effect with indigenous rhizobia.

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## Solubilizacija neorganskih fosfata endofitima *Pseudomonas* sp. iz nodula boranije

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**Sažetak:** Fosfor (P) je jedan od esencijalnih makronutrijenata potrebnih za rast biljaka. Fosfosolubilizirajuće bakterije (PCB) spadaju u grupu heterotrofnih bakterija sposobnih da solubilizuju fosfor iz nerastvornih organskih i neorganskih jedinjenja, oslobađajući fosfor koji je dostupan biljkama. U ovom radu su ispitivane endofite bakterije izolovane iz nodula boranije gajene u različitim sistemima interkropinga. Samo 14 od 158 endofitnih izolata je solubilizovalo trikalcijum fosfate (TCP). Nijedan izolat nije solubilizovao  $\text{FePO}_4$  i  $\text{AlPO}_4$ . Osam izolata je formiralo zone solubilizacije šire od 3 mm i selektovano je za dalji rad. TCP solubilizacija je kvantifikovana u tečnom Pikovskaya medijumu i iznosila je 14-182  $\mu\text{gml}^{-1}$  rastvorenog fosfora. TCP solubilizacija je praćena zakišeljavanjem medijuma, a izmereno je opadanje pH sa 7,4 na 5,57 i 4,91 kod izolata sa najmanjim i najvećim solubilizacionim potencijalom. Selektovan je najefikasniji izolat MP18 i identifikovan kao *Pseudomonas* sp. prema izvršenoj analizi sekvence 16S rRNK gena.

**Cljučne reči:** endofite bakterije, fosfosolubilizirajuće bakterije, noduli boranije, *Pseudomonas*, zemljišni pH