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ORIGINAL PAPER

Performance evaluation of potent phosphate solubilizing bacteria in potato rhizosphere

Mohammad Ali Malboobi · Mandana Behbahani · Hamid Madani · Parviz Owlia · Ali Deljou · Bagher Yakhchali · Masoud Moradi · Hassan Hassanabadi

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Abstract Three phosphate solubilizing bacterial isolates identified as *Pantoea agglomerans* strain P5, *Microbacterium laevaniformans* strain P7 and *Pseudomonas putida* strain P13 were assessed for mutual relationships among them, competitiveness with soil microorganisms and associations with plant root using *luxAB* reporter genes for follow-up studies. Synergism between either *P. agglomerans* or *M. laevaniformans*, as acid-producing bacteria, and *P. putida*, as a strong phosphatase producer, was consistently observed both in liquid culture medium and in root rhizosphere. All laboratory, greenhouse and field experiments proved that these three isolates compete well with naturally occurring soil microorganisms. Consistently, the combinations of either *P. agglomerans* or *M. laevaniformans* strains with *Pseudomonas putida* led to higher biomass and potato tuber in greenhouse and in field trials. It is conceivable that combinations of an acid- and a phosphatase-producing bacterium would allow simultaneous utilization of both inorganic and organic phosphorus compounds preserving the soil structure.

Keywords Phosphate solubilization · *Pantoea agglomerans* · *Microbacterium laevaniformans and Pseudomonas putida* · Biofertilizer · Potato

M. A. Malboobi
Applied Microbiology Research Group, Jahad Daneshgahi, Tehran University, Tehran, I.R. Iran

M. A. Malboobi (✉) · B. Yakhchali
National Institute of Genetic Engineering and Biotechnology, P.O. Box 14155-6343, Tehran, I.R. Iran
e-mail: malboobi@nigeb.ac.ir

M. Behbahani · A. Deljou
Department of Biotechnology, Faculty of Agriculture, Bu-Ali-Sina University, Hamedan, I.R. Iran

H. Madani
Department of Agriculture, Arak Islamic Azad University, Arak, I.R. Iran

P. Owlia
Department of Microbiology, Shahed University, P.O. Box 14155-7435, Tehran, I.R. Iran

M. Moradi
Kesht-Va-Sanat Moradi Inc., Arak, I.R. Iran

H. Hassanabadi
Potato and Onion Department, Seed and Plant Research Institute, Karaj, I.R. Iran

Introduction

Beneficial bacteria, collectively called plant growth promoting rhizobacteria (PGPR), are the main constituents of biofertilizers. These bacteria promote plant growth and health by various means such as mineralization of nutritional elements, nodulation and nitrogen fixation (Zhang et al. 1996), synthesizing phytohormones such as hormones (Khalid et al. 2004), microbial iron transport agents or siderophores (Kloepper et al. 1980), antibiotic production against plant pathogens (Sandra et al. 2001; Morales et al. 2008), suppressing pathogens or combinations of them (Somers and Vanderleyden 2004). Nevertheless, the performance of PGPR as biofertilizers is severely influenced by both biotic and abiotic environmental conditions of the target regions.

While total phosphorus contents of soil is typically high, the available phosphate ions (P_i), the prevalent forms of phosphorus that plant roots absorb, is usually suboptimal (for a review see Rodriguez and Fraga 1999). Therefore, P_i-demanding crops such as potato, *Solanum tuberosum*, rely on the use of high amounts of chemical P_i fertilizer.

Alternatively, Pi solubilizing bacteria (PSB), as a group of PGPR, facilitate the hydrolysis of a wide range of phosphorus compounds leading to higher crops yields and reduce chemical hazards to the environment.

In this study, competitiveness and P_i solubilizing activities of three PSB isolates were investigated in liquid culture and in soil using genetically-labelled bacteria. Also, the effects of each isolate and mixed inocula on potato growth and yield were examined in both greenhouse and field conditions. The primary goal of these experiments was to determine the efficacies of the isolates to be used as biofertilizers.

Materials and methods

Reporter gene transfer

Three PSB isolates used in this study were as described by Malboobi et al. (2009). In order to follow bacterial strains in mixed cultures, the isolates were labelled with *nptII* gene, as a selective marker and *luxAB*, as reporter genes. First, each PSB was subjected to increasing concentrations of rifampin (12, 25, 50, 75 and 100 μg/ml) to select for spontaneous mutations. The rifampin resistant (Rif^R) subpopulations were used for conjugation with a donor bacterium, *Escherichia coli* W803, carrying pDLB30 plasmid (Boivin et al. 1988) kindly provided by Dr. Hani Antoun (Laval University, Ste-Foy, Québec, Canada). *Trans*-conjugation procedure was as described (Chabot et al. 1996). Those bacteria that were stably resistant to kanamycin but sensitive to chloroamphenicol were selected as *trans*-conjugants through at least 10 alternative passages in medium with and without 50 μg/ml kanamycin plus100 μg/ml rifampin. Luminescence was observed by adding one drop of N-decyl aldehyde (Sigma) to the top lids of plates (Chabot et al. 1996). In all cases, no luminescent bacterium was detected prior to the addition of substrate.

Competition assays

Competition assays for each *trans*-conjugant alone or in combination with other bacteria (non-labelled PSB or soil-extracted bacteria) were done in liquid medium. Soil-extracted bacterial suspension was prepared through adding one gram of a soil sample into 10 ml of sterilized ddH_2O and mixed vigorously. After precipitation of soil, 1 ml of supernatant containing typical mixture of soil bacteria was used. For the assays, 120-ml Erlenmeyer flasks containing 15 ml of Sperber medium containing approximately 10^4 CFU/ml of each bacterial strain was incubated at 30°C and 120 rpm overnight. The same non-inoculated medium served as control in each case. Time-coursed measurements for growth index (GI) were estimated by colony count and P_i solubilizing index (PSI) in culture supernatant was measured by Fisk and Sabbarow method (1925).

Greenhouse experiment on potato

Tissue-cultured potato seedlings derived from nodal meristems were planted into pots containing sterile or non-sterile soil uniformly mixed with 30 ml suspension of either individual or combinations of two or three PSB containing 10^8 CFU/ml of each strain while only one bacterial strain was labelled in each case. Pots were arranged in a completely-randomized design with three replicates. Growth conditions were a photoperiod of 8 h dark and 16 h light with maximum intensity of florescent light of 2,000 μmol m^{-2} s^{-1}, an average temperature of 28°C, a mean humidity of 60% and irrigation every day for 2 months. After 25 days, samples were taken from roots or soils at 2 or 5 cm away from the stem base for Lux^+ bacterial counts. At the end of experiment, plants were harvested by washing roots with tap water. Roots and shoots were air dried and incubated at 75°C for 48 h prior to the measurements of dry weights.

Field trials on potato

Field trials were carried out in two different climatic conditions with high-P_i soil (28.4 mg/kg; Arak, Markazi Province, Iran) and medium-P_i soil (11.6 mg/kg; Karaj, Tehran Province) during the growing season of year 2002. In both locations, treatments were arranged in a complete randomized block design with three replicates. In these experiments, all combinations of main factors levels, ammonium phosphate fertilizer (0, 50 and 100 kg/ha) and bacterial biofertilizers (no bacteria, *M. laevaniformans* and *P. putida*, *P. agglomerans* and *P. putida*, and *M. laevaniformans*, *P. agglomerans* and *P. putida*) were examined. Potato seed tubers were soaked directly in a bacterial suspension containing 10^8 CFU/ml of each strain. At tuber stage, leaves were sampled to measure phosphorus contents. At the end of the season, the yield was calculated based on the weight of tubers collected for 20 plants per plot. MSTAT-C software version 2.10 (Michigan State University, Michigan) and Microsoft Excel program were used for computational analysis of the data. Means were compared using Duncan's new multiple-range test at $P < 0.05$.

Results

Having screened soil samples collected from a diverse range of slightly alkaline soil types, three competent PSB were isolated. Two isolates identified as *Pantoea*

agglomerans strain P5 and *Microbacterium laevaniformans* strain P7 hydrolyzed insoluble phosphate effectively. Another isolate, *Pseudomonas putida* strain P13, showed the highest phosphatase activity (Malboobi et al. 2009). In order to make PSB distinguishable from other bacteria in the mixed cultures, kanamycin resistance and luminescence phenotypes were conferred to the isolates by transferring *nptII* and *luxAB* genes through *trans*-conjugation. Bacterial competence for conjugation differed greatly such that the rates were as high as 10^{-6}, 10^{-4} and 10^{-6} for P5, P7 and P13 strains, respectively. All selected *trans*-conjugant derivatives were similar to their respective wild type parents as judged by GI and PSI curves (data not shown).

In a series of competition assays, each labelled bacterium was cultured individually, with the other PSB or with soil microorganisms. Although no strong antagonism was observed among these bacterial strains grown on solid LB medium (data not shown), however, minor inhibition of growth was obvious for some combinations in liquid medium as shown in Fig. 1. For instance, the GI of P5 and P7 was slightly lower in the co-cultures than in the single cultures. Similarly, the growth rate of P5 was affected negatively in the presence of P13 while the reverse was not true. In the triple cultures, GI of all bacterial strains was lower than when each strain was cultured separately. Interestingly, there was not noticeable difference in PSI of single, double or triple cultures, except for the co-culture of P5 and P7 strains (Fig. 1d). When isolated PSBs were cultured in the presence of soil microorganisms, GI and PSI values for all strains were relatively lower. This was more pronounced for P13 strain (Fig. 1).

To assess the performance of PSB isolates in the presence of soil microorganisms particularly in rhizosphere, tissue-cultured potato seedlings were grown in pots in which single or combined cultures of bacteria were mixed uniformly with either sterile or non-sterile soils. While all inoculated roots appeared luminescent due to the presence of high population of Lux^+ PSBs (not shown), bacterial counts for P5 and P7 strains did not vary significantly in samples taken 2 or 5 cm away from the plant roots (Fig. 2a, b). In contrast, GI of the P13 in 2- or 5-cm samples were statistically different based on mean comparison at $P < 0.05$ (Fig. 2c), indicating a close association between root and this bacterium.

We have also measured root and shoot dry weight of 2-month old plants treated with various PSB inoculants at the end of experiment. Table 1 shows the means of shoot and root dry weight and ranked based on mean comparison at $P < 0.05$. In line with the conclusions derived from GI and PSI values of mixed PSB cultures (Fig. 1), the highest

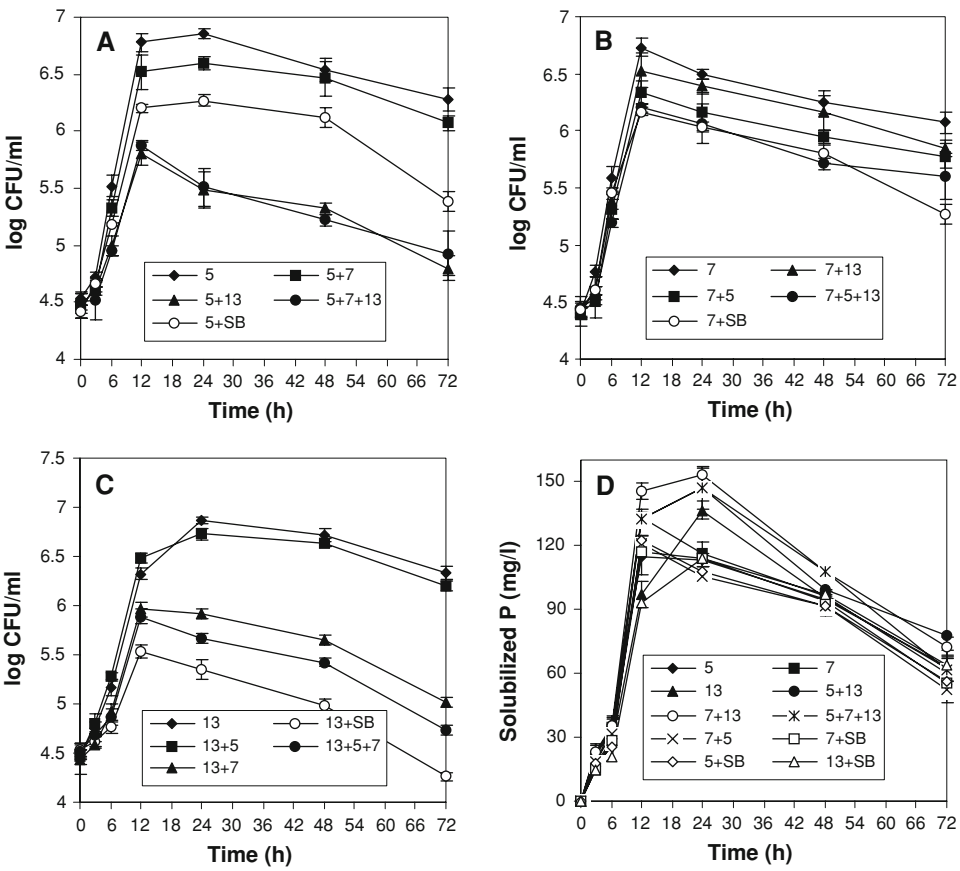

Fig. 1 Competitiveness of phosphate solubilizing bacterial (*PSB*) isolates assayed in minimal liquid medium. Growth of labelled *Pantoea agglomerans* strain P5 (**a**), *Pseudomonas putida* strain P7 (**b**) and *Microbacterium laevaniformans* strain P13 (**c**) as well as phosphate solubilisation rates (**d**) were measured when grown either individually, in the presence of other PSBs or along with typical soil bacteria (*SB*). All data points are the means of three replicates. Standard errors are shown by *vertical bars*

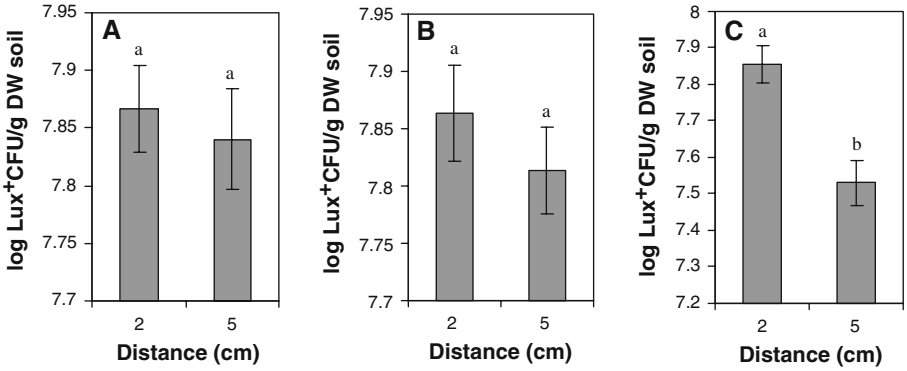

Fig. 2 Bacterial distribution around the roots of pot-grown potato plants. The population size of Lux^+ *Pantoea agglomerans* strain P5 (**a**), *Pseudomonas putida* strain P7 (**b**) and *Microbacterium laevaniformans* strain P13 (**c**) in the soil samples at 2 or 5 cm away from the roots are given as log of colony counts. All data points are the means of three replicates. Standard errors are shown by *vertical bars*

Table 1 Mean comparisons of shoots and roots dry weights of treated pot-grown potato inoculated with each bacterial isolates and their combinations in the absence or presence of soil microorganisms (non-sterile soil)

Treatments	Shoot dry weight (g)	Root dry weight (g)
P5 + P13	0.351 a	0.0526 a
P7 + P13	0.325 ab	0.043 ab
P5 + P7 + P13	0.269 cb	0.04 bc
P13	0.25 cb	0.03 cd
P7 + non-sterile soil	0.24 cd	0.026 de
P7	0.22 cde	0.023 def
P5 + non-sterile soil	0.196 cde	0.0193 efg
P5	0.195 cde	0.018 efg
P13 + non-sterile soil	0.16 ed	0.012 fgh
No bacterium	0.153 e	0.007 gh
P5 + P7	0.145 e	0.0047 h

Means marked with the same letters are not significantly different according to Duncan's new multiple range test ($P < 0.05$). Treatments are as described in "Materials and methods". P5, *Pantoea agglomerans* strain P5; P7, *Pseudomonas putida* strain P7; and P13, *Microbacterium laevaniformans* strain P13

shoot or root biomass was gained with inoculum containing both P5 and P13 which was followed by a mixture of P7 and P13. The lowest growth rate was obtained for the inoculum of combined P5 and P7, which was even less than that of control plants.

Similarly, statistical analysis of the data gathered from the field trials revealed that potato tuber yields were affected significantly by the main factors, ammonium phosphate levels or PSB treatments, and their interactions at both locations (Table 2). In comparison, the ammonium phosphate levels and different PSBs had no significant effects on phosphorus contents of plant leaves at the tuber stage in the high- and medium-Pi soil types. However, significant differences were obtained for the interactions of variables which varied from 0.65 to 0.75% depending on treatments (Table 2).

In both locations, the highest tuber yield was gained when the amount of ammonium Pi was reduced to one half and an inoculum containing both P5 and P13 strain was used (50–59.4 t/h). The lowest yield of potato tuber was obtained for treatments with P5 and P7 bacteria or with no fertilizer. Despite considerable difference between two locations, combined analysis of variance reconfirmed the positive effects of P5 plus P13 inoculum (data not shown).

Discussion

As shown by the other authors (Boivin et al. 1988), resistance to kanamycin and bioluminescence are appropriate genetic markers for follow-up experiments. The use of transformed bacteria alone or in combination with the other PSB isolates as well as soil microbiota revealed the interactions among the relevant populations in the minimal medium. Except for low levels of growth inhibition in some cases, no antagonistic effect was noticed between the examined bacterial species (Fig. 1a–c). While this study was done in minimal culture medium resembling soil nutritional constituents, we have seen similar situation in rich medium too (data not shown). No notable changes in PSI values in single and multiple cultures suggests that P_i solublizing is a function of total bacterial counts as they act synergistically in this regard.

Since the conditions in soils are much more complex than those in vitro, further studies on the root-colonizing competence of the isolates were necessary. Although all PSB were somewhat dependant on root exudates, only the association of P13 strain with rhizosphere was statistically significant. Kuiper et al. (2002) also found *P. putida* in close association of roots with prevalent dependence on root exudates. The available data indicate that the movement and attachment of *P. putida* to root surface enhanced by nutrient limitation (Gu and Mazzola 2001). The attachment of *P. putida* to bean root occurs via a glycoprotein named agglutinin (Buell and Anderson 1992). On the bacterial side, antigenic lipopolysaccharide (LPS)

Table 2 Mean comparison between leaf phosphorus content and potato yield in two experimental locations, Karaj and Arak

Treatments	Karaj		Arak	
	P content (%)	Potato yield (t/h)	P content (%)	Potato yield (t/h)
APF				
No P_i	0.71 a	40.2 b	0.69 a	45.6 b
50	0.71 a	42.5 a	0.69 a	50.0 a
100	0.70 a	41.2 ab	0.68 a	48.0 a
PSB				
No PSB	0.68 a	41.4 b	0.70 a	46.7 b
P7 + P13	0.70 a	41.4 b	0.70 a	42.5 c
P5 + P13	0.70 a	48.0 a	0.72 a	49.7 a
P5 + P7 + P13	0.70 a	33.2 c	0.70 a	47.0 b
Interaction of APF and PSB				
No P_i, no PSB	0.66 bc	49.0 abc	0.70 bc	46.1 bcde
No P_i, P7 + P13	0.68 b	45.2 cde	0.72 b	44.3 cde
No P_i, P5 + P13	0.70 a	45.0 cde	0.75 a	38.7 e
No P_i, P5 + P7 + P13	0.65 bc	37.0 e	0.69 c	53.4 ab
50, no PSB	0.69 ab	40.0 de	0.71 b	49.5 bc
50, P7 + P13	0.70 a	49.2 abc	0.69 c	44.1 cde
50, P5 + P13	0.70 a	50.0 ab	0.7 bc	59.4 a
50, P5 + P7 + P13	0.68 b	42.0 de	0.7 bc	47.0 bcd
100, no PSB	0.71 a	46.0 cde	0.71 b	44.5 cde
100, P7 + P13	0.72 a	40.0 de	0.71 b	39.2 de
100, P5 + P13	0.68 b	50.0 ab	0.70 bc	51.0 bc
100, P5 + P7 + P13	0.68 b	40.0 de	0.70 bc	40.6 de

Means marked with the same letters are not significantly different according to Duncan's new multiple range test ($P < 0.05$). The effects of main factors, ammonium phosphate fertilizer (APF, kg/ha; top section) and phosphate solubilizing bacteria (PSB; middle section), and their interactions (bottom section) are shown. Treatments are as described in "Materials and methods". P5, *Pantoea agglomerans* strain P5; P7, *Pseudomonas putida* strain P7; and P13, *Microbacterium laevaniformans* strain P13

molecules are accounted for mediating the interaction with root cells (de Weger et al. 1996). Mutants lacking the relevant LPS showed impaired colonization ability (Rodriguez-Herva et al. 1999).

In both greenhouse and field experiments, all inoculants of individual PSB and their combinations, except for P5 plus P7 mixture, led to increased growth rates of potato plants (Tables 1, 2). Even though we have seen no strong antagonism between colonies of these two isolates (data not shown), inhibition of growth in the mixed liquid culture as well as decrease in potato root and shoot biomass accumulation could be due to secretion of some metabolites. The minimal effect of soil microorganisms on GI of PSB could be attributed to competition for nutrients since higher yield was evident for inoculated non-sterile soils (Table 1). Interestingly, the P_i levels of soil or application of chemical P_i fertilizer did not cause much difference in potato yield. Nevertheless, when PSB inoculate, P5 plus P13 in particular, was added; the yield was increased for 20–25% (P2B3 vs. P1B1; Table 2).

In conclusion, the criteria of the isolated PSB are sufficiently superior for being introduced as P_i biofertilizers. Preliminary pot experiments and subsequent field trials indicated the advantageous use of double inoculants of *Pseudomonas* and *P. agglomerans* or *M. laevaniformans* strains on the growth rate of potato plants. These were consistent with the outcomes of many other field trials conducted for several crops within the last 5 years that would be reported elsewhere.

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