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Uptake of phosphate and promotion of vegetative growth in glucose-exuding rice plants (*Oryza sativa*) inoculated with plant growth-promoting bacteria

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ABSTRACT

We measured phosphorus uptake by rice plants inoculated with plant growth-promoting bacteria (PGPB) using *Pseudomonas* sp. strain PAC, *Serratia* sp. strain CMR165, and *Azospirillum brasilense* strain FT326. We measured plant growth parameters and phosphate solubilization and uptake. Results show that the ability to solubilize phosphates varied among PGPB strains. Strain FT326 was unable to solubilize phosphates. In the presence of glucose, PAC and CMR165 can solubilize inorganic tricalcium phosphate and organic calcium magnesium inositol hexaphosphate. Phosphate solubilization by strains PAC and CMR165 was different over time; FT326 was similar to the untreated control. Plants inoculated with PAC or CMR165 had higher concentrations of phosphates than those inoculated with FT326 and plants that were not inoculated. Glucose was the only sugar identified in rice root exudates. PAC and CMR165 promoted plant growth and uptake of phosphate and could be used as biofertilizers to optimize phosphate fertilization.

1. Introduction

After nitrogen, phosphorus (P) is the most limiting nutrient for plant growth. While abundantly present in many soils, it is immobilized by fixation in the form of insoluble Ca, Fe, or Al salts or Al and Fe oxides not available to plants (Rodríguez and Fraga, 1999; Richardson et al., 2001). Even in fertile soils, P concentration in the soil solution is not higher than 10 μM at pH 6.5 (Arnou, 1953). This phenomenon is dependent on pH and soil type (Dey, 1988).

According to Goldstein et al. (1993), P accumulated in agricultural soils can supply maximum yields of crops for 100 years. Approximately, 80% of inorganic P applied as fertilizer is quickly immobilized in the soil, becoming unavailable for plant absorption (Holford, 1997). Organic P in soil varies from 30% to 50% of total P in most soils. This fraction is formed mainly by inositol-phosphate (phytate) which is the most stable organic form of P (Dalal, 1977). Other types of organic P are phosphoesters, mainly of high molecular weight, that need to be converted to low-weight soluble forms to be readily available for plants (Goldstein, 1994).

Plant responses to P deficiency tend to increase its availability, absorption, and efficiency of use. Adaptation mechanisms to increase Pi availability include phosphatases, RNAases, and nuclease activities (for organic phosphates) and organic acid production (for inorganic phosphates) (Rodríguez et al., 2006). Root adaptations to low levels of P include mycorrhizal associations, alterations of root architecture, an increase in length and density of root hairs (Raghothama, 1999; Raghothama and Karthikeyan, 2005), and enhanced expression of high affinity phosphate transporters to increase P absorption (Kochian, 2000).

Conceptually, plant growth-promoting bacteria (PGPB) can affect plant growth and development either directly or indirectly (Bashan and Holguin, 1998). On the one hand, PGPB may decrease or prevent some effects of phytopathogenic organisms by producing antibiotics. On the other hand, these bacteria may directly provide plants with different compounds or facilitate incorporation by fixing nitrogen or solubilizing phosphorus. PGPB can solubilize phosphates effectively and enhance P uptake in poor, calcareous P soils or those fertilized with phosphoritic rock (Kundu and Gaur, 1984; Magda et al., 2003).

Microbial activity is one of the most important pathways of solubilizing P in soils (Halder et al., 1989; Gyaneshwar et al., 2002; Rodríguez et al., 2006). Among PGPB are phosphate-solubilizing bacteria (PSB). The phenotype exhibited by PSB is attributed to lower pH caused by low-molecular-weight organic acids or proton liberation to the medium (Gyaneshwar et al., 1998). Organic acids can dissolve Pi directly through anionic interchange or chelating ions associated with the Pi (Goldstein, 1994). Production of organic acids has been identified in PSB (Rodríguez and Fraga, 1999; Vyas

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; HPLC, high performance liquid chromatography; IP6, calcium magnesium inositol hexaphosphate; PGPB, plant growth-promoting bacteria; PSB, phosphate-solubilizing bacteria; TCP, tricalcium phosphate.

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and Gulati, 2009). These acids are produced in the periplasm of many Gram-negative bacteria through a direct oxidation pathway of glucose (Liu et al., 1992).

In turn, plants supply root-borne carbon compounds, mainly sugars that can be metabolized for growth of bacteria (Goldstein, 1994). Lynch and Whipps (1990) estimated that 4–29% of photosynthates can be transferred to the rhizosphere and available to microorganisms. Root exudates are important nutrients for soil microorganisms and are involved in chemotactic root colonization processes (Lynch and Whipps, 1990).

PGPB colonize the rhizosphere and adhere to the root surface (Kloepper and Schroth, 1978; Bashan and Holguin, 1998; Kloepper et al., 1999) or penetrate the roots to establish endophytic populations (Gray and Smith, 2005).

To convert organic P into forms readily available for plants, it must be mineralized into low-weight inorganic forms. Organic P is mineralized by microbial phosphatases (Bishop et al., 1994), whose enzymatic activities were detected in different types of soils (García et al., 1992).

This study measured phosphorus uptake by rice plants using isolated and characterized PGPB from Argentine soils and compost in our laboratory. Our objective was to assess the degree of phosphate solubilization and plant growth-promotion of isolated bacterial strains. We hypothesize that in the sole presence of insoluble phosphates, plant growth is promoted when the plants are inoculated with phosphate-solubilizing strains of bacteria.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *Pseudomonas* sp. strain BNM 0521 (PAC) was isolated from compost of green leaves of *Platanus hispanica* Mill. After appropriate steps of isolating this bacteria, it was cultured to obtain pure cultures in Cetrimide agar. The *Serratia* sp. strain BNM0522 (CMR165) was isolated from rice roots. To isolate bacteria, plant roots were washed with distilled water and 1 cm fragments from the main roots were cut and incubated in NFb semisolid broth with malate as the carbon source. After incubation for 48 h at 33 °C, the number of CFU was determined by serial dilution plating on Congo Red nutrient agar (Rodríguez-Cáceres, 1982). Morphology and motility were determined for each isolate. Biochemical tests were also performed; the isolated bacteria were selected for its capacity to produce indol acetic acid (IAA) and nitrogenase activity according to Torres et al. (2000) and Ribaudo (personal communication).

Azospirillum brasilense strain FT326 (FT326) was provided by the Brazilian Enterprise for Research on Agriculture (EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária) and used as a negative control strain. Strains were grown in NFb with malate as the carbon source, supplemented with NH_4Cl (1 g l^{-1}), at 35 °C under continuous agitation (100 rpm) for 48 h (Döbereiner et al., 1976).

2.2. Molecular characterization

Isolates were grown at 25 °C for 24 h in BHA; after lysing cells, the DNA was extracted using a microbial DNA isolation kit (Ultra Clean, Mo Bio Laboratories, Carlsbad, CA). The DNA was checked for purity using standard methods (Sambrook et al., 1989). DNA templates were amplified in a thermocycler (Genius, Techne, Staffordshire, UK), using universal primers amplifying a 1000 bp region of the 16S rDNA, 616F: 5′-AGA GTT TGA TYM TGG CTC AG-3′, 699R: 5′-RGG GTT GCG CTC GTT-3′ (Invitrogen, Carlsbad, CA). These primers are located at positions 8–25 and 1099–1113 (*Escherichia coli* numbering), respectively. The amplification mixture (100 μl) contained 2 μl (50 pmol μl^{-1}) each of the 616F and 699R primers, 0.5 μl (2 U μl^{-1}) of Taq DNA polymerase (F-5495, Finnzymes, Vantaa, Finland), 10 μl 5× reaction buffer (F-5495, Finnzymes, Vantaa, Finland), 10 μl dNTP mixture containing 1 mmol l^{-1} each of dATP, dGTP, dCTP, and dTTP (F-5605, Finnzymes, Vantaa, Finland), 70 μl sterile, filtered water (Milli-Q purification system, EMD Millipore, Billerica, MA) and 100 ng DNA template. Reactions were run for 10 min at 94 °C followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Controls devoid of DNA were included in the amplification process. The integrity of the PCR products was controlled through the development of a single band after electrophoresis in 2% (w/v) agarose gels in TBE buffer at 5 V cm^{-1} for 1 h. Amplicons were purified using an UltraClean PCR clean-up kit (Mo Bio Laboratories), and subsequently sequenced, using an automated sequencer (Abi Prism 3730, Applied Biosystems, Carlsbad, CA) using a cycle sequencing kit, premixed format (BigDye Terminator v3.1, Applied Biosystems). Sequencing primers were the same as those used in the amplification reaction, but diluted ten times (5 pmol l^{-1}).

The resulting 16S rDNA sequences were compared in a BLAST search with those in the National Library of Medicine database (Altschul et al., 1997). The 16S rDNA sequence for strain PAC was submitted to GenBank (accession number FJ851181) and compared with known sequences using BLASTN PROGRAM (Zhang et al., 2000); search showed a 99% identity for 16S ribosomal RNA gene of *Stenotrophomonas maltophilia* strain ZA-6 (accession number: FJ851181.1) and *Pseudomonas aeruginosa* strain PS1 (accession number FJ705886.1). The strain CMR165 was submitted to GenBank (accession number FJ851180); search showed a 99% identity with *Serratia marcescens* strain AGPim1A 16S ribosomal RNA gene (accession number JF683415.1).

2.3. Bacterial solubilization and phosphatase activity of TCP and IP6

The ability of isolates to solubilize phosphate was assessed qualitatively on agar plates containing an insoluble P (Goldstein and Liu, 1987). Bacterial suspensions (100 μl $OD_{600\ nm}$ = 0.2 in sterile 0.85%, w/v NaCl) were inoculated in a well in agar medium containing (g l^{-1}) NH_4Cl (5); NaCl (1), $MgSO_4 \cdot 7H_2O$. Malate, sucrose, lactose (5) or glucose (10) as alternative carbon sources and either $Ca_3(PO_4)_2$ or IP6 (5) mg ml^{-1} as insoluble P were added to the medium source in suspension. The pH was adjusted to 7.2. Plates were incubated at 37 °C for 48 h. Development of a clear zone around the colony was regarded as a positive indicator of P solubilization. To evaluate $[H^+]$, dependent P solubilization, methyl red (as a pH indicator) and 100 mM Tris–HCl buffer at pH 7.9 were added to the medium.

Stationary bacterial culture suspensions (2.5 ml, $OD_{600\ nm}$ = 0.2 in sterile 0.85%, w/v NaCl) were inoculated in Erlenmeyer flasks with 50 ml fresh broth (Liu et al., 1992). Flasks were incubated at 37 °C and agitated at 100 rpm. To compare bacterial and acid P solubilization, 0.5 m 1 M HCl was added to the medium to reach a similar pH to the inoculated cultures (Kim et al., 1997). At 0, 24, 48, 72, and 96 h after inoculation, 1 ml aliquots (duplicates) were centrifuged at 8000 × *g* for 10 min. The pellet was discarded and the $[H^+]$ (colorpHast, EMD Industries, Gibbstown, NJ) and soluble P were determined in the supernatant (Fiske and Subbarow, 1925). Phosphatase activity was determined according to Pond et al. (1989) in plates containing agar Luria Bertani and 200 μg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP).