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Short communication

Phosphate-solubilizing *Pseudomonas putida* can influence the rhizobia–legume symbiosis

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Abstract

Allfalfa and soybean are the most important leguminous plants in the agricultural system of the semiarid pampas of Argentina. The possible action of phosphate solubilizing bacteria on the leguminous–rhizobia symbiosis was studied since in this region the available phosphorus distribution is not uniform. The strains used were *Sinorhizobium meliloti 3DOh13*, a good solubilizer of iron and phosphorus for alfalfa, *Bradyrhizobium japonicum TIIIB* for soybean and two phosphorus-solubilizing strains of *Pseudomonas putida* (*SP21* and *SP22*) for growth promotion treatments. Modification of shoot and root system dry weights occured in soybean but not in alfalfa in presence of *Pseudomonas* strains.

Keywords: Phosphate-solubilizing bacteria; *Pseudomonas*; Rhizobia; Nitrogen fixation; Co-inoculation

Phosphorus is an essential plant nutrient which is added to soil as soluble inorganic phosphate that, in a large portion, becomes insoluble and, therefore, unavailable to plants (Sing and Kapoor, 1994). Furthermore, this mineral is one of the most affected by the degrading processes of the soils.

The most abundant phosphates in the semiarid Argentinean Pampas are bound to calcium (Buschiazzo et al., 1994). The continuous agriculture during more than 20 years caused considerable decreases in the soluble phosphorus and in smaller degree in the total inorganic phosphorus (Urioste et al., 1996).

Numerous microorganisms, especially those associated with roots, have the ability to increase plant growth and productivity. Since the early observations of a better growth of plants inoculated with phosphate-solubilizing bacteria, different results have pointed to the ability of soil bacteria to solubilize phosphorus from rock phosphate during culture in synthetic nutrient media or in association with living plants (Azcon et al., 1976; Illmer et al., 1995).

The insoluble inorganic compounds of phosphorus can be converted by bacteria into available phosphates for plant roots. Improvement of acid production in the rhizosphere as the result of bacteria inoculation is certainly involved in phosphate solubilization by both bacteria and plants. The main active strains in this conversion belong to a range of genera, including *Pseudomonas*, *Mycobacterium, Micrococcus*, *Bacillus*, *Flavobacterium*, *Rhizobium*, *Mesorhizobium and Sinorhizobium* (Asea et al., 1988; Salih et al., 1989, Rodríguez and Fraga, 1999). Bashan and Holguin (1997) showed that the beneficial effects of *Azospirillum* on plants can be enhanced when co-inoculated with other microorganisms. They observed that co-inoculation increased both growth and yield (compared to the single inoculation) and also improved the absorption of nitrogen, phosphorus and mineral nutrients. Thus, plant growth can be increased by dual inoculation with *Azospirillum* and phosphate-solubilizing bacteria.

El-Komy (2005) demonstrated the beneficial influence of co-inoculation of *Azospirillum lipoferum* and *Bacillus megaterium* for providing balanced nitrogen and phosphorus nutrition of wheat plants.

The inoculation with bacterial mixtures provided a more balanced nutrition for the plants and the improvement in

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root uptake of nitrogen and phosphorus was the major mechanism of interaction between plants and bacteria (Belimov et al., 1995).

There is evidence that some *Pseudomonas* species increase nutrient absorption, as N, P and K, in addition to act as biocontrol agents of phytopathogenic fungi and produce phytohormones in the rhizosphere, which promote plant growth (O'Sullivan and O'Gara, 1992). *Pseudomonas putida* strains have been cited as phosphate solubilizers (Kumar and Singh, 2001, Villegas and Fortin, 2002).

Alfalfa (*Medicago sativa* L.) is the most important forage legume in the semiarid Argentinean Pampas because of the quality nutrients that it provides (Viglizzo, 1995). Furthermore, the effect that this plant has on soil fertility is very important, as well as the contribution of its root system to the improvement and conservation of the soil structure (Vance, 1997). Soybean (*Glycine max* L.) cultivation is continuously expanding in Argentina and its growth in surface and production has been accelerating in the last decade, converting this country in one of the principal world exporters (Lattanzi, 2002).

The objective of this study was to evaluate the effect of two phosphate solubilizing *Pseudomonas* strains on the symbiosis of rhizobia with alfalfa and soybean.

Rhizobia strains used were *Sinorhizobium meliloti 3DOh13*, *Bradyrhizobium japonicum TIIIB*, *P. putida SP21* and *P. putida SP22*. All these bacteria belong to the collection of our group of investigation in the Universidad Nacional de Río Cuarto, Argentina.

Rhizobia were routinely grown on yeast extract mannitol (YEM) solid medium (Vincent, 1970) and Pseudomonads on tryptic soy agar (TSA) medium (Britania Laboratory, Argentina).

For the phosphate solubilization assay, we used a medium containing 2 g yeast extract, 20 g glucose, 2 g tricalcium phosphate, 60 mg actidione, 15 g agar made up to 1000 ml with water, at pH 7. This medium was inoculated with the relevant strains and incubated at 28 °C for 5 days. Bacterial colonies forming clear zones were considered to be phosphate solubilizers.

Additionally, we tested the production of siderophores by all strains. The Chrome azurol S method described by Alexander and Zuberer (1991) was used. Plates were incubated at 28 °C for 5 days, and colonies exhibiting an orange halo were considered to be siderophore producers.

Soybean seeds were disinfected for 20 min with 0.4% calcium hypochlorite solution and alfalfa seeds were scarified by shaking for 15 min in concentrated sulfuric acid, then disinfected with 70% ethanol for 3 min. Seeds were then washed with several changes of sterile, distilled water.

For inoculation assays, seeds were transferred under aseptic conditions onto the surface of perlite/sand (2:1) bed and allowed to germinate. Two days after sowing, seeds were inoculated with its corresponding rhizobia strain and with the *Pseudomonas* strains. Bacterial cultures were obtained in the described media, following standard procedures cointaining 10^9 CFU ml^{-1} for rhizobia and 10^6 CFU ml^{-1} for *Pseudomonas* adjusted by optical density. Two milliliters of each inoculum were applied to the root system of each seedling in the planting hole.

Plants were watered alternately with sterile, distilled water and a modified N-free Jensen solution (Vincent, 1970) where the P source was changed by tricalcium phosphate.

Uninoculated controls were watered in the same manner, but with the addition of 0.5% KNO_3 l^{-1} to the original Jensen solution (control with soluble phosphate) and to the Jensen's modified solution with tricalcium phosphate (control with insoluble phosphate).

Plants were grown in a chamber under controlled conditions: 16 h day at 28 °C and 8 h night at 16 °C, and a light intensity of 220 μE m^{-2} s^{-1}. Forty days after sowing, plants were harvested in order to evaluate nodulation and dry weight. Each treatment was performed in three independent experiments with three replicates per treatment.

Results were handled statistically by analysis of variance (ANOVA). When ANOVA showed significant treatment effects, LSD test was applied at 0.05 level of significance.

S. meliloti 3DOh13 solubilized iron and phosphate while *B. japonicum TIIIB* was a poor phosphate solubilizer and siderophore producer. *P. putida SP21* and *SP22* mobilized iron and phosphate with greater efficiency than *B. japonicum TIIIB* and *S. meliloti 3DOh13*, with mobilization zones greater to 15 mm (Table 1).

A greater number of nodules and dry weight was registered in soybean when the co-inoculation with *B. japonicum TIIIB* and *Pseudomonas* was completed (Table 2).

Differences were not observed with respect to inoculation with *S. meliloti* alone when alfalfa was co-inoculated with *S. meliloti 3DOh13* and *Pseudomonas* (Table 3).

Result can be explained by the fact that *Pseudomonas* provides phosphorus when growing with *B. japonicum TIIIB* but not with *S. meliloti 3DOh13*. The phosphate solubilization by *S. meliloti 3DOH13* would be sufficient for plant growth.

The presence of *P. putida* strains did not negatively affect the rhizobia symbiosis.

Table 1
Phosphate solubilization and siderophore production of bacterial strains

Strain	Phosphate solubilization (diameter of halo in mm)	Production of siderophores (diameter of halo in mm)
S. meliloti 3DOh13	8.2±0.4	5.0±0.7
B. japonicum TIIIB	1.0±1.2	2.2±0.5
P. putida SP21	18.1±2.7	16.1±0.9
P. putida SP22	21.0±3.1	17.5±1.3

Results represent the mean of three replications per strain.