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SHORT COMMUNICATION

Response of alfalfa (*Medicago sativa* L.) to single and mixed inoculation with phosphate-solubilizing bacteria and *Sinorhizobium meliloti*

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Abstract The objectives of this work were to phenotypically and genetically characterize alfalfa rhizosphere bacteria and to evaluate the effect of single or mixed inoculation upon nodulation and biological nitrogen fixation. Thirty-two strains showed tricalcium phosphate solubilization ability, and two of them caused bigger or equal solubilization halos than the control strain *P. putida* SP22. The comparison of the 16S ribosomal DNA sequences indicated that these strains are phylogenetically related to *Bacillus* spp. and *Pseudomonas* spp. A beneficial effect of both isolates on alfalfa growth was observed in coinoculation assays. *Pseudomonas* sp. FM7d caused a significant increase in root and shoot dry weight, length, and surface area of roots, number, and symbiotic properties of alfalfa plants. The plants coinoculated with *Sinorhizobium meliloti* B399 and the *Bacillus* sp. M7c showed significant increases in the measured parameters. Our results indicating that strains *Pseudomonas* sp. FM7d and *Bacillus* sp. M7c can be considered for the formulation of new inoculants.

Keywords Alfalfa · Plant growth-promoting rhizobacteria · Coinoculation · Biological nitrogen fixation

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Introduction

Phosphorus (P) is an essential nutrient for plants that is added to soil as soluble inorganic phosphates that, generally, become insoluble and cannot be used by crop plants (Singh and Kapoor 1994). Therefore, P is often a limiting nutrient in agricultural soils.

Several bacterial species, associated to plant rhizosphere, can cause beneficial effects upon plants (Glick 1995) and for this reason, are nominated plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). They can include some phosphate-solubilizing bacteria that are used as biofertilizers in agriculture (Subba Rao 1993; Rodríguez and Fraga 1999). The insoluble inorganic compounds of phosphorus can then be converted into plant usable forms. Acid production by these rhizosphere bacteria is certainly involved in phosphates solubilization (Richardson 2001; Chen et al. 2006). The main strains able to perform this conversion belong to the genera *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Achromobacter*, *Erwinia*, *Agrobacterium*, *Burkholderia*, *Flavobacterium*, *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* (Rodríguez and Fraga 1999; Fernández et al. 2007).

Coinoculation of leguminous seeds with nitrogen-fixing symbiotic bacteria and plant growth-promoting genera has acquired an increasing interest (Glick 1995) because of the

positive effects on leguminous growth including early nodulation, an increase in the number of nodules, higher nitrogenase activity, and root respiration, a superior uptake of water and nutrients by the roots and a higher general plant health (Knight and Langston-Unkefer 1988; Sindhu et al. 2002; Rosas et al. 2006). Coinoculation with *Azospirillum* and phosphate-solubilizing bacteria increased growth, yield, and uptake of nitrogen, phosphorus, and other minerals of sorgum and barley plants (Bashan and Holguin 1997).

In Argentina, alfalfa (*Medicago sativa* L.) has traditionally been the basis of livestock production and together with wheat, one of the founding crops of Argentinean agriculture. At the present time, it constitutes one of the main forage resources because of its enormous adaptation to different soils and climates. Moreover, this crop is important in sustaining the structure and nitrogen fertility of soils in which it grows, when it is correctly associated to specific rhizobia strains. Therefore, it required the development of biotechnological products that combine microbial inoculants (multiple inoculants) stimulating an optimal biological nitrogen fixation (BNF) and uptake of water and nutrients by crop. The objective of this study was to evaluate the effect of single or mixed inoculation of two phosphate-solubilizing bacterial isolates and *Sinorhizobium meliloti* upon growth, nodulation, and BNF efficiency in alfalfa.

Materials and methods

Isolation of bacteria from soil

The isolates were obtained from soil samples of the experimental field of the Instituto Nacional de Tecnología Agropecuaria (INTA), located in Manfredi, Argentina (Córdoba province, 63°44′W 31°50′S) and Faja Maisan, Chile (72°55′W 39°05′S). Neither alfalfa was cropped nor other *S. meliloti* was inoculated in the last 5 years in these soils. Some soil properties are shown in Table 1. One gram of each soil was suspended into 9 ml of sterile saline solution (9 g l^{-1} NaCl). Serial 10-fold dilutions were performed. Then, a 0.1 ml aliquot was plated in 25% tryptic soy agar (TSA; Britania®) in triplicate. Plates were incubated at 28°C for 24–48 h. Results were expressed as colony forming units/g soil (CFU/g soil). Isolated colonies were maintained in 25% TSA medium.

Characterization of bacterial isolates

The first characterization consisted in the direct observation of the isolated colonies, taking into account the color, shape, elevation, margins, diameter, surface, opacity, and texture (Zinniel et al. 2002). Each isolate was subjected to gram stain and, additionally, to the complementary 3% KOH test (Suslow et al. 1982).

The ability of the bacterial isolates to solubilize tricalcium phosphate was tested on a medium containing 2 g yeast extract, 20 g glucose, 2 g tricalcium phosphate, 60 mg actidione, and 15 g agar made up to 1,000 ml with water at pH 7 (Rosas et al. 2006). This medium was stab inoculated with the relevant strains and incubated at 28°C for 5 days. Experiments were performed in triplicate. Bacterial colonies forming clear zones were considered to be phosphate solubilizers. Positive control was made with *Pseudomonas putida* SP22 (Rosas et al. 2006) in similar culture conditions.

Genotypic identification was performed by amplification and partial nucleotide sequencing of the 16S ribosomal DNA (16S rDNA) of the isolates that showed an equal or higher phosphate solubilization capacity than the control. The partial nucleotide sequences of the 16S rRNA gene (rDNA) were determined by direct sequencing of appropriate PCR products. A DNA region corresponding to nucleotides 20 to 338 of *Escherichia coli* 16S rDNA was amplified from each strain with the universal primers Y1 (59-TGG CTC AGA ACG AAC GCT GGC GGC-39) and Y2 (59-CCC ACT GCT GCC TCC CGT AGG AGT-39) as previously described for proteobacteria (Young et al. 1991). The 25 µl PCR mixtures contained: 0.5 µM of each primer, 200 µM dNTPs, 3 mM $MgCl_2$, PCR reaction buffer (50 mM KCl, 20 mM Tris HCl at pH 8.0), 1 U Taq DNA polymerase (Promega Corp.), and 2 µl of template DNA, previously obtained by heating a freshly isolated bacterial colony in 50 µl of distilled water to 100°C for 15 min. The amplifications were carried out in thermo cycler (I Cycler-BioRad). The cycling conditions were as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 20 s, at 52°C for 20 s, at 72 °C 45 s, and at 72°C for 2 min.

Table 1 Some chemical and physical characteristics of Faja Maisan and Manfredi soils

Country/location	Percentage of organic matter	pH	P (µg/g)	K (cmol/kg)	N (µg/g)	Ca (cm/kg)	Mg (cm/kg)	Na (cm/kg)	Soil type	Cropping history
Chile/Faja Maisan	17.0	5.4	14.0	1.23	37.0	4.52	1.27	0.13	Typic hapludand	Mixed prairie
Argentina/ Manfredi	1.71	6.2	46.0	ND	10.5	ND	ND	ND	Haplustol	Grain crops

ND not determined

After the reaction, 10 μl of the PCR reaction were analyzed in 1.5%-agarose gels containing 1 μg/ml of ethidium bromide and photographed with a Kodak DC290 Digital camera. The nucleotide sequence of the PCR products was determined for both strands with an automatic laser fluorescent DNA Sequencer (Macrogen, Korea). The partial sequences of the 16S rDNA genes have been deposited in the GenBanK under the accession numbers: GQ 373390 and GQ 373391 and compared against the complete database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Single and mixed inoculation assay

The characterized isolates were tested for their capacity to coexist with *S. meliloti* B399 (strain recommended by INTA for alfalfa inoculation in Argentina) in 25% TSA and yeast extract mannitol agar (YEMA) (Vincent 1970). Strain were streaked each on one side of a Petri plate, and plates were incubated at 28°C for 48–72 h. Rhizobacteria that showed normal growth in the presence of the rhizobial strain in both culture media were selected for further assays involving single or mixed inoculation.

Each phosphate-solubilizing strain and *S. meliloti* were used to prepare a small amount of inoculant using steam-sterilized Tierra del Fuego peat (pH 6.5), which was conditioned according to the method described by Roughley (1970) and saturated with each of the strains of interest grown in 25% tryptic soy broth. Growth curves were performed in order to standardize the appropriate bacterial inoculum for each microbial species according to Koch (1981). The inoculant formulation was carried out with an approximate concentration of 10^8–10^9 viable cells per g. The inoculants were allowed to mature in test tubes (Somasegaran and Hoben 1994) at room temperature for 7 days and then kept at 4°C. Colony counts were performed during formulation and seed inoculation.

Alfalfa seeds (*Medicago sativa* L.) cv. Bárbara SP (INTA Manfredi) were scarified for 10 min in concentrated sulfuric acid (Suttie 2000), washed ten times with sterile distilled water (SDW) for 3 min, surface disinfected with 70% ethanol for 1 min, and washed three times with SDW. Then, 2 g of seeds were coated with 1 g of each inoculant (approximately 10^3–10^4CFU/g seeds) or with a 1:1 mixture of inoculants (phosphate-solubilizing isolate and *Sinorhizobium*). Eight seeds were sown in polyethylene pots (6.5 cm diameter, 15 cm height) containing a soil:sand:perlite (2:1:1) sterile mix.

The assay included the following treatments: single inoculation with *S. meliloti* B399, single inoculation with the phosphate-solubilizing isolate, and mixed inoculation with *S. meliloti* B399 and the phosphate-solubilizing isolate. Pots were kept in a growth chamber at 28±2°C with light–dark cycles (16 h light and 8 h dark) under a light intensity of 220 μE/m^2/s, and plants were alternatively watered with SDW and a modified N free Jensen (Vincent 1970) in which the original P source was replaced by tricalcium phosphate. We included two uninoculated controls, one with the addition of KNO_3 0.005 g/l to the Jensen solution during irrigation (CN) and other without nitrogen added (C).

The plants inoculated only with a phosphate-solubilizing isolate were watered in the same manner as CN. Growth parameters were evaluated 45 days after sowing.

Shoot and root length, shoot, root and nodules dry weights, root surface area (Carley and Watson 1966), and number of nodules (and their position in the roots) were determined for each treatment.

Determination of nitrogenase activity

The acetylene reduction technique (Hardy et al. 1968) was used for nitrogenase activity determination. The nodules from each plant were placed in 10 ml vials, and 10% of their atmosphere was substituted with acetylene. After 30 min

Table 2 BLAST comparison between partial 16S ribosomal gene of selected local isolates

Sample	Description	E value	Identity	GenBank number[a]
FM7d (GQ 373391)[a]	*Pseudomonas teessidea*, PR65T	3 e-148	100%	AM419154.2
	Pseudomonas sp. DK6	3 e-148	100%	EU158320.1
	Pseudomonadaceae bacterium FND-2	3e-148	100%	EF017809.1
	Pseudomonas sp. NZ039	3e-148	100%	AY014808.1
	Uncultured bacterium, clone FD03B06	3e-148	100%	FM873410.1
M7c (GQ 373390)[a]	*Bacillus* sp. AiL3	3e-153	100%	FJ860227.1
	Bacillus sp. XL62	3e-153	100%	FJ465166.2
	Bacillus licheniformis strain H3	3e-153	100%	FJ713021.1
	Bacillus velezensis	3e-153	100%	AB244463.1
	Bacillus amyloliquefaciens strain BCRC 14193	4e-152	99%	EF433408.1

[a] GenBank accession numbers of the sequences analyzed in this paper

Table 3 Effect of single and mixed inoculation of alfalfa with *Bacillus* spp., *Pseudomonas* spp., and *Sinorhizobium meliloti* B399 on root dry weights (mg), shoot dry weights (mg), root length (cm), and shoot N content (mg g^{-1})

Treatment	Root dry weight (mg)	Shoot dry weight (mg)	Root length (cm)	Root surface area (cm^3)	Shoot N (mgg^{-1})
Uninoculated control	1.80a	2.03a	3.61a	0.16a	41.9ab
Uninoculated control + N	1.51a	3.01a	3.38a	0.18a	46.2b
Bacillus spp.	2.18a	2.53a	3.49a	0.28b	41.5a
Pseudomonas spp.	1.64a	2.24a	3.53a	0.25b	41.9ab
S. meliloti B399	1.59a	3.94b	3.56a	0.32b	45.6b
S. meliloti B399 +*Bacillus* spp.	3.15b	4.18b	4.56b	0.44c	49.6c
S. meliloti B399 +*Pseudomonas* spp.	3.69b	4.58b	4.83b	0.64d	42.7ab

Different letters within the same column indicate significant differences according to DGC test ($P<0.05$)

incubation, 100 μl of the sample was injected in a Hewlett Packard 5890 Series II plus gas chromatograph at constant pressure to determine the produced ethylene. The column used was Parapak N (polyvinylpyrolidone), 1/8, 2 mm D. I., which allowed the analysis of gases of compounds containing up to six carbons.

Determination of total N content

This determination was done by a modified Kjeldahl method in a semimicro scale (Baker and Thompson 1992) by treating 100 mg sample with 1.25 g of catalytic mix (potassium sulfate:mercuryc oxide, in a 24:1 relation) and 2.5 ml of concentrated sulfuric acid. The mixture was digested for 40 min. Then, the ammonia liberated by alkalinization with NaOH solution was separated by distillation and collected (as ammonium borate) in a 4% boric acid solution. Ammonium was determined by titration with 0.02 N HCl with an automatic analyzer of nitrogen and Kjeltec Auto 1030 (Tecator, Sweden) was used.

Statistical analyses

Results were analyzed statistically by analysis of variance using the InfoStat computer software (Universidad Nacional de Córdoba, Argentina 2000). When analysis of variance showed significant treatment effects, the Di Rienzo–Guzmán–Casanoves (DGC) test was applied to make comparisons among the means at the 0.05 level of significance.

Results

Bacterial count was about 10^6 CFU/g dry soil for the different soils. In this study, 32 phosphate-solubilizing rhizobacteria were isolated. Two of them, M7c and FM7d, were selected because of their phosphate solubilization activity since the zone of solubilization (halo) was greater than that of the reference strain *P. putida* SP22 (2.5 and 2.0 cm respectively); M7c and FM7d also showed to coexist with *S. meliloti* B399 in YEMA and 25% TSA. In order to further characterize the identity, the two selected strains, a PCR of the 16S fragment, was carried out obtaining a 297 bp fragment for M7c and a 288 fragment for FM7d. Pairwise comparisons were made between homologous 16S gene segments and showed that the 16S rDNA sequences of isolates M7c and FM7d shows highest levels of identity (100–99%) with *Bacillus* sp. and *Pseudomonas* sp., respectively (Table 2).

Plant coinoculated with *S. meliloti* B399 and *Pseudomonas* spp. had a greater root system than plants coinoculated with *S. meliloti* B399 and *Bacillus* spp. and consequently, a greater root dry weight. Root length of the plants coinoculated with phosphate-solubilizing bacteria and *S. meliloti* B399 was greater than that of other treatments (Table 3).

The coinoculated alfalfa plants showed a greater foliar development than plants inoculated with a microbial species and the uninoculated plants. The shoot dry weight of coinoculated plants was statistically significant than those of the uninoculated plants and plants inoculated with a phosphate-solubilizing strain, whereas, the shoot dry

Table 4 Effect of single and mixed inoculation of alfalfa with *Bacillus* spp., *Pseudomonas* spp., and *Sinorhizobium meliloti* B399 on nodule number ($plant^{-1}$), nodules dry weight (mg), and nitrogenase activity (μmoles ml ethylene g nodule $fresh^{-1}$ h^{-1})

Treatment	Nodule number ($plant^{-1}$)	Nodules dry weight (mg)	Nitrogenase activity (μmoles ml ethylene g nodule $fresh^{-1}h^{-1}$)
S. meliloti B399	2.2a	0.17a	1.17a
S. meliloti B399 +*Bacillus* spp.	2.8a	0.18a	1.19a
S. meliloti B399 +*Pseudomonas* spp.	4.6b	0.41b	1.22a

Different letters in column indicate significant differences according to DGC test ($P<0.05$)

weight of plants inoculated with *S. meliloti* B399 was not statistically different (Table 3).

The N content of the alfalfa plants inoculated with *S. meliloti* B399 and *Bacillus* spp. was significantly the greatest, whereas, the N content of plants inoculated with *S. meliloti* and *Pseudomonas* spp. was lower than those of the uninoculated plant (treated with KNO_3) and plants inoculated with strain *S. meliloti* B399 (Table 3).

Shoot length was greater in plants inoculated with a single phosphate-solubilizing bacteria and uninoculated plants (data not shown), whereas, there were no significant differences between the coinoculated plants and those treated with *S. meliloti* B399.

A significant increase in number of nodules and nodules dry weight was observed in the plants inoculated with *S. meliloti* and *Pseudomonas* spp. compared to plants inoculated with *S. meliloti* B399 (Table 4). The percentage of nodules was about 85%, and the calculation of relative frequencies showed that coinoculated plants had high proportion of nodules in the secondary roots. The single inoculation with *S. meliloti* B399 only showed nodules in the primary root (data not shown). Nitrogenase activity was detected in all nodules of the studied plants. However, no statistically significant differences were detected (P= 0.1884) between single and mixed inoculations (Table 4).

Discussion

Two of phosphate-solubilizing bacteria (M7c and FM7d strains) showed a greater solubilizing capacity than that of the control strain *P. putida* SP22 under in vitro conditions. These isolates were identified as *Bacillus* spp. (M7c) and *Pseudomonas* spp. (FM7d) by PCR amplification and partial nucleotide sequencing of the ribosomal 16S DNA. Numerous reports indicate that the species of the genera *Pseudomonas* and *Bacillus* possess the ability of solubilizing phosphate (Illmer and Schinner 1992; Chen et al. 2006; Wani et al. 2007). Phosphorus is an essential nutrient being involved in several metabolic processes and being a component of important molecules in alfalfa (Mikkelsen 2004). Azcon et al. (1988) reported that a correct P supply increases alfalfa yield, number and size of nodules, and N fixation. Inoculation of millet and pea with *B. circulans* or *B. megaterium* var. *phosphaticum* increased both yield and P uptake, in growth chamber studies (Saber et al. 1977; Raj et al. 1981). Similarly, Gaind and Gaur (1991) reported that inoculation of mung with *B. subtilis* increased grain yield, and uptake of P and N when soil was amended with rock phosphate.

Mixed inoculants provide better nutritional balance for plants and improvements in N and P uptake were the main mechanisms involved (Rodríguez and Fraga 1999). The coinoculation of soybean with *B. japonicum* and the phosphate-solubilizing bacteria *Pseudomonas striata* shown an increase in dry weight of nodules and dry matter yield (Wasule et al. 2003). This could be because the rhizobacteria stimulate the growth of rhizobia in soil through the production of various compounds (Bashan 1999).

It has also been observed that some PGPR strains are capable of stimulating additional infection sites that are then occupied by the rhizobia (Plazinski and Rolfe 1985). Coinoculation of alfalfa with *Pseudomonas* sp. FM7d or *Bacillus* sp. M7C and *S. meliloti* B399 significantly increased most of the measured parameters, probably related to the increase in plant P uptake by plants coinoculated. Our results agree with those of other authors who reported the positive effect of *Pseudomonas* and *Bacillus* on plant growth (Bolton et al. 1990; Dashti et al. 1998; Sindhu et al. 1999; Wani et al. 2007)

The isolates selected in this work showed a beneficial effect on alfalfa growth, especially in shoot biomass, which is used for forage and shepherding. The use of microorganisms as inoculants is of great interest especially in areas with low P availability (Deubel and Merbach 2005; Jorquera et al. 2008). This technology would minimize the negative impact of chemical fertilizers on the environment and promote sustainable agriculture in Argentina.

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