Plant Soil (2014) 385:357–371
DOI 10.1007/s11104-014-2237-z

REGULAR ARTICLE

Growth, nutrient uptake and symbiosis with rhizobia and arbuscular mycorrhizal fungi in *Lotus tenuis* plants fertilized with different phosphate sources and inoculated with the phosphate-solubilizing bacterium *Pantoea eucalypti* M91

Luis Nazareno Castagno · Ileana Vanesa García · Analía Inés Sannazzaro · Matías Bailleres · Oscar Adolfo Ruiz · Rodolfo Ernesto Mendoza · María Julia Estrella

Received: 2 January 2014 / Accepted: 12 August 2014 / Published online: 21 August 2014

Abstract
Background and aims The aim of this work was to evaluate the ability of P fertilization and phosphate-solubilizing bacteria (PSB) inoculation to promote the growth of *L. tenuis* in typical soils of the Salado River Basin (Argentina) with low P availability.
Methods Aboveground biomass and P and N levels were evaluated in field-grown *L. tenuis* plants inoculated with *Pantoea eucalypti* M91, either without fertilization or in combination with phosphate rock and triple superphosphate (TSP). The impact of P fertilization and inoculation on the symbiotic interactions between *L. tenuis* and native rhizobia bacteria and arbuscular mycorrhizal fungi was also evaluated.
Results Inoculation with M91 increased the *L. tenuis* biomass production and P concentration in shoots, at an early stage of plant growth. The combined treatment of inoculation with M91 and TSP significantly increased the P and N content in shoots compared to non-inoculated plants, fertilized or not. *P. eucalypti* M91 was found to endophytically colonize roots and leaves of *L. tenuis* plants grown in vitro and also under field conditions.
Conclusions The results suggesting that inoculation of *L. tenuis* with the PSB such as *P. eucalypti* M91 strain might allow more efficient use of N and P and a more sustainable option for grasslands producers from the Salado River Basin, in order to reduce costs and avoid increased levels of P insoluble in soils.

Keywords *Lotus tenuis* · P sources · Phosphate-solubilizing bacteria · Soil P availability · Rhizobia · Arbuscular mycorrhizae

Responsible Editor: Katharina Pawlowski.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-014-2237-z) contains supplementary material, which is available to authorized users.

L. N. Castagno · A. I. Sannazzaro · O. A. Ruiz · M. J. Estrella (✉)
IIB INTECH (UNSAM-CONICET),
Av. Intendente Marino Km 8,200 CC 164 (7130), Chascomús, Buenos Aires, Argentina
e-mail: estrella@intech.gov.ar

I. V. García · R. E. Mendoza
Museo Argentino de Ciencias Naturales Bernardino Rivadavia (MACN), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET),
Av. Ángel Gallardo 470, (C1405DJR), Chascomús, Buenos Aires, Argentina

M. Bailleres
Chacra Experimental Integrada de Chascomús (CEICH) (MAA-INTA),
Mitre 202. (7130), Chascomús, Argentina

Introduction

Frequently, chemical P fertilizers are applied to soils in order to meet P demands from crops, but they become rapidly transformed into immobile forms through

precipitation reactions with aluminum or iron in acidic soils, and with calcium in normal or calcareous soils (Hao et al. 2002; Gyaneshwar et al. 2002). Under these conditions, the availability of soluble P-forms for plant growth becomes strongly limited. As an alternative to P fertilization, many soil microorganisms that play a key role in soil P dynamics can increase P uptake by plants. Inorganic P forms can be solubilized by phosphate-solubilizing bacteria (PSB), which excrete organic acids that dissolve phosphate minerals and/or chelate cationic partners of the PO_4^{3-} anion,, thus releasing P into the solution (Vyas and Gulati 2009). Although these bacteria can be used as plant growth promoters, usually their amounts in soil are not enough to compete with other well-established bacteria in the rhizosphere, making it necessary to increase their levels by plant inoculation with adequate strains (Igual et al. 2001). In this sense, a pre-requisite for introducing these beneficial bacteria in the environment is, together with the ability to promote plant growth, to be adapted to the soil conditions and successfully compete with the soil microflora (Taurian et al. 2010). In this regard, the isolation and characterization of native bacteria could provide adequate strains for crop inoculation in particular soils where these microorganisms are well adapted.

The Salado River Basin is a typical breeding area for beef production in which perennial grasses predominate over other plant species, a common scenario in natural temperate regions. Thus, either as a consequence of land use or selective grazing, a decline or even absence of native perennial legumes is evident in this region (Montes 1988). In this way, the promotion of native or naturalized legumes present in these grasslands is an alternative to increase forage quality and yield. *Lotus tenuis* (=*L. glaber* Mill.), a perennial legume of European origin that was naturalized in the Salado River Basin, is highly valuable in this region because of its contribution to the forage offer and its positive influence on growth of associated species (Díaz et al. 2005). However, the successful implantation of this legume in the grassland community is usually limited by its low growth-rate during early developmental stages, which strongly decreases its ability to compete with weeds (Blumenthal and McGraw 1999). As a consequence, cultural practices that improve *L. tenuis* implantation are expected to increase forage yield and quality. Plant growth is restricted by several adverse factors in the Salado River Basin, such as alternating periods of water deficit and excess, as well as saline and alkaline soils with low nutrient content (Garcia and Mendoza 2008). In particular, soils in this region exhibit low P availability for plant growth, which seriously affects forage quality and productivity (Escudero and Mendoza 2005; Garcia and Mendoza 2008). In this way, inoculation of *L. tenuis* with selected PSB native from soils in this region is expected to improve P acquisition, thus improving growth of this legume and associated grasses. This approach could then be an affordable and sustainable practice to improve forage production in the Salado River Basin. In this regard, it should be kept in mind that in addition to being an essential nutrient for all plant species, P is particularly important for legumes due to the high ATP demand of the symbiotic nitrogen fixation process established with soil rhizobia (Robson et al. 1981). Therefore, this is an additional reason why the consequences of inoculating *L. tenuis* plants with PSB in the Salado River Basin are worth to be explored.

In a previous study aimed to improve the quality of bacterial inoculants for *L. tenuis* in the Salado River Basin, we isolated and characterized PSB from the rhizosphere of this legume. Thus, 17 gram-negative PSB belonging to different genera such as *Pantoea, Erwinia, Pseudomonas, Rhizobium* and *Enterobacter* were identified. Among them, *Pantoea eucalypti* isolate M91 exhibited the highest efficiency in promoting growth of *L. tenuis* plants through P solubilization, as shown by experiments in culture media containing $Ca_3(PO_4)_2$ (non-soluble) as the sole P source. Thus, this was the first report about plant growth promotion by *P. eucalypti*, as well as the first report about promotion of *L. tenuis* growth by inoculation with PSB (Castagno et al. 2011).

The aim of the present work was to evaluate the ability of P fertilization and PSB inoculation to promote growth of *L. tenuis* in typical soils of the Salado River Basin. For this purpose, aboveground biomass, P and N levels were evaluated in field-grown *L. tenuis* plants inoculated with *P. eucalypti* M91, either without fertilization or in combination with phosphorous fertilizers of different solubility levels. Phosphate rock and triple superphosphate were used as low and high solubility phosphate sources, respectively, both of which were applied at a moderate concentration (20 kg P ha^{-1}). The impact of P fertilization and *P. eucalypti* inoculation on the symbiotic interactions between *L. tenuis* and

native rhizobia bacteria and arbuscular mycorrhizal (AM) fungi was also evaluated.

Materials and methods

Soil features and site location

The experimental plots were established in the Chacra Experimental Integrada de Chascomús (*Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires - Instituto Nacional de Tecnología Agropecuaria*) located in Chascomús, Argentina (35° 30′ S, 58° 30′ W) during the period 2010–2011. Local air-temperature and rainfall data collected over 75 years (1911–1986) were provided by the *Centro de Investigaciones Biometeorológicas* (CIBIOM-CONICET) and were used to compare the temperature and rainfall recorded during the experimental period at the Experimental Station. The experimental plot was located in a typical area devoted to cattle breeding for beef production, sustained by natural pastures dominated by perennial and seasonal grasses, which lack traditional legumes such as red and white clover (*Trifolium pratensis* and *T. repens*) and lucerne (*Medicago sativa*). The experiment was conducted in a soil classified as a typical Natraquoll (Wermbter and Ramallo 1980) and its main chemical properties were pH: 6.7, EC (electrical conductivity): 2.4 dS m^{-1}, C (organic carbon): 2.02 %, N (total nitrogen): 0.23 %, OM (organic matter): 3.5 %, P-availability (Bray I): 11.6 ppm, ESP (exchangeable sodium percentage): 13.2 % in the 0.20 m upper A1 horizon, followed by a 0.23 m B2t horizon with pH: 9.2, EC: 1.7 dS m^{-1}, C: 0.36 %, N: 0.04 %, OM: 0.62 %, P Bray: 3.5 ppm P, ESP: 51 %.

Inoculum preparation and seed inoculation

The phosphate-solubilizing bacterium *P. eucalypti* M91 used in this work was isolated from the rhizosphere of *L. tenuis* growing in a typical lowland of the Salado River Basin and further characterized by biochemical and molecular studies (Castagno et al. 2011). In order to prepare the inoculant, *P. eucalypti* M91 strain was grown in liquid TY (Sperry and Wilkins 1976) at 30 °C on a rotatory shaker (180 rpm). Bacterial cultures were started in 3 ml medium and after 24 h they were sub-cultured into 250 ml flasks containing 50 ml of fresh culture medium. After an additional 24 h-incubation period, cultures were inoculated into 2 L flasks containing 500 ml of fresh medium and further incubated for 48 h. Then, this culture (1×10^9 bacteria /ml) was half-diluted with sterile water and used for seed inoculation, by spreading the bacterial suspension over the sown line..

Experimental design

The experimental area was sprayed with glyphosate twice, 40 days before sowing and a few days after sowing, in order to eliminate weeds. Additionally, Flumetsulam (Preside®) was applied after sowing and before emergence to eliminate broadleaf plants. Before sowing, the land was leveled by a disc harrow to homogenize sowing depth. After leveling, *L. tenuis* cv. Esmeralda was sown on August 24th (winter) in plots 9.0 m long and 1.5 m wide at a rate of 17 kg ha^{-1}. Plots were fertilized with either triple superphosphate (TSP, 20 %P) or phosphate rock (PR, 13 %P) from Gafsa (Tunisia) at an equivalent rate of 20 kg P ha^{-1}, or were not fertilized (−P). Two weeks after sowing, plots were divided in two subplots (4.5 × 1.5 m) and (PSB) *P. eucalypti* M91 was inoculated in solution by irrigating one of the two subplots of fertilized and non-fertilized plots. The experimental model was a randomized complete block trial in a split-plot design where the main plots corresponded to the P-sources (PR, TSP and -P) and the subplots to the inoculation treatment (inoculated and control (non-inoculated)). Treatments were replicated four times and the whole experiment thus consisted of 24 subplots.

Re-isolation of *P eucalypti* M91 from *L. tenuis* plants grown under controlled conditions

With the aim of assessing whether *P. eucalypti* M91 is able to endophytically colonize *L. tenuis* plants, seedlings derived from surface-disinfected seeds were inoculated with the above-mentioned strain and cultivated under controlled conditions, according to the methodology described by Sannazzaro et al. (2011). Endophytic colonization of *L. tenuis* plants by *P. eucalypti* M91 was evaluated by epifluorescence microscopy. For this purpose, leaves from inoculated and non-inoculated plants were surface-disinfected with 70 % (v/v) ethanol for 2 min and afterwards were immersed in a solution of acridine orange (0.1 %, pH 7.0) for 3 min, which allows visualization of both bacterial cells and anatomical

structures of the leaf surface (Monier and Lindow 2004). Leaves were then air-dried and placed on top of a molten water-agar layer (1 %) previously spread on a microscope slide, to ensure a flat surface for microscopic observation. Samples were analyzed with a Nikon Eclipse E600 microscope equipped with a 450 to 490 nm excitation filter and a 520 to 560 nm emission filter. Images were acquired with a Nikon DS-Qi1Mc video camera.

Re-isolation and identification of *P. eucalypti* M91 from *L. tenuis* plants

In order to confirm the endophytic colonization of *L. tenuis* plants by *P. eucalypti* M91 under field conditions, endophytic PSB were isolated from roots and shoots at the second harvest. These organs were obtained from inoculated and non-inoculated plants and were surface disinfected as previously described. Plant material was then homogenized in 0.85 % NaCl and serial dilutions of the extracts thus obtained were plated on NBRIP agar medium (Nautiyal 1999) and incubated 48–72 h at 30 °C. Colonies showing a clear halo of phosphate solubilization were used to obtain DNA by the method described by Sannazzaro et al. (2011). BOX and REP-PCR patterns of the strains thus isolated were obtained and compared to those of *P. eucalypti* M91.

Aboveground biomass, plant and soil analyses

Three harvests were performed at different time points during the 261-day-period of the experiment: December 14th 2010, February 28th 2011 and May 11th 2011. Samples were taken from a 0.25-m^2 area randomly chosen within the central rows of each sub-plot. Aboveground plant material was collected clipping 0.03 m above the soil surface. After each harvest, the experimental area was mown to 0.03 m and the aboveground material was removed from the plot.

Additionally, three plants were withdrawn from each subplot along with their rhizospheric soil. Roots were separated from the soil by washing in deionized water, and soil was used to analyze pH (1:2.5 suspension in water), EC, available P (Kurtz and Bray 1945), C (Richter and von Wistinghausen 1981), and total N (Bremmer and Mulvaney 1982).

Aboveground plant material obtained as described above was dried at 70 °C for 48 h and weighed. Yield was then calculated as plant biomass (kg) per hectare. P and N levels were determined by the molybdovanadophosphoric acid and Kjeldahl methods, respectively (Jackson 1964). For P determination, dry aboveground-plant material was previously digested in a nitric-perchloric acid mixture (3:2). For N determination, plant material was previously digested in sulphuric acid.

AM colonization and nodulation of *L. tenuis* roots by rhizobia

Mycorrhizal root colonization was measured in plants collected from each subplot, by using fresh roots cleared in 10 % KOH for 12 min at 90 °C and stained in 0.05 % lactic acid–glycerol Trypan blue (Phillips and Hayman 1970). Three plants per plot and twenty-five root segments per plant were examined under a microscope (×200). The fraction of root length colonized (MC), and the root length containing arbuscules (AC), vesicles (VC), and hyphae only (HO) was determined following McGonigle et al. (1990), and the entry points per mm of colonized roots was measured by the method of Amijee et al. (1989). The nodules formed on *L. tenuis* roots by native rhizobial populations of the experimental field were counted under a binocular stereomicroscope (×7.5).

Determination of AM fungal spore density in rhizospheric soil

Rhizopheric soil samples (250 g) were taken from each subplot and kept in plastic bags at 4 °C until used for the quantification of AM-fungal-spore density. AM fungal spores were isolated from soil by a modification of the sucrose gradient centrifugation technique (Daniels and Skipper 1982) and counted under ×35 magnification using a dissecting microscope. Spore density was expressed as the number of spores per 100 g of dry soil.

Statistical analyses

Data sets were tested for normality and heterogeneity of variance (heteroscedasticity) with standard methods. Non-normal data were appropriately transformed to compare treatment means. Analyses of variance (split-plot design) were used to test the equality of treatment means. Mean separation was performed by the LSD test. Treatment effects from data that could not be normalized by any of the common standard transformations were tested with the Kruskal-Wallis non-parametric test. Statistix 9.0 software was used for statistical analyses.

Results

Climate and soil

The soil used for the field experiment represented a typical condition of the Salado River Basin area, with a top horizon close to neutrality followed by a B2t horizon very poor in nutrients and OM, strong alkalinity with 51 % of ESP and 45 % of clay plus fine silt (Wermbter and Ramallo 1980). The drainage capacity was limited, and the soil was not suitable for crop production. During the experimental period (August–May), mean air temperature was 16.7 °C and accumulated rainfall was 524 mm. Air temperature was quite similar to the historical temperature recorded at the same location for 75 years (1911–1986), but the accumulated rainfall was 35 % lower (Fig. S1). Excepting for September, January and February, monthly rainfall was markedly lower before the first (October–December) and the third harvest (March–April), as compared to the historical recorded rainfall.

Effect of P fertilization and PSB inoculation on the aboveground biomass

P. eucalypti M91 inoculation exerted a highly significant effect on dry matter yield at the first harvest (P=0.0001, Tables 1 and 2), as opposed to the second and third harvests (P=0.6216 and P=0.0989 respectively, Tables 1 and 2). In this way, inoculated plants exhibited a 68 % increase in dry matter yield at the first harvest, as compared to non-inoculated controls (Table 1). P fertilization had no effect on biomass yield at any harvest (Tables 1 and 2). Analysis of variance also revealed that the interaction between P fertilization and inoculation with PSB was not significant in terms of dry matter yield at any harvest (Table 2). The multiple comparison of results obtained at the first harvest showed a tendency to increase biomass production of inoculated plants, regardless of P addition and the P source used (Table 1). A significant increase in biomass production was obtained in inoculated, non-fertilized plants or fertilized with PR, as compared to their respective non-inoculated controls (Table 1).

Inoculation of *L. tenuis* with *P. eucalypti* M91 significantly affected accumulated dry matter yield (P=0.0104, Table 2), and P-source had no effect on accumulated dry yield (P=0.0893, Table 2). A multiple comparison of accumulated biomass showed a similar tendency to that observed in the first harvest, evidenced as an increase in the biomass of inoculated plants, as compared to non-inoculated ones regardless of P fertilization (Fig. 1a).

Effect of P-fertilization and PSB inoculation on shoot-P and N levels

In order to evaluate the impact of PSB inoculation and P fertilization on plant nutrition, P and N levels were analyzed in plant shoots. P concentration was significantly affected by PSB inoculation at the first and second harvests (P=0.0012 and P<0.0001, Tables 1 and 2). Thus, P concentration of PSB-inoculated plants was 15.4 and 13.3 % higher than non-inoculated plants, for the first and second harvests, respectively (Table 1).

A multiple comparison of the results obtained for the first harvest showed that P concentration of PR- and TSP-fertilized and inoculated plants increased by 7.7 and 14.3 %, as compared to their respective non-inoculated controls (Table 1). For this harvest, a similar tendency was observed for inoculated plants that were not fertilized, although differences in P concentration observed between inoculated and non-inoculated plants were not statistically significant (Table 1). At the second harvest, P concentration was higher in inoculated than in non-inoculated plants (Table 1). In this way, inoculation increased P concentration by 28.6 % in TSP-fertilized plants, 10.3 % in PR-fertilized plants and 9.4 % in non-fertilized plants (Table 1). At the third harvest, no differences in P concentration were detected between inoculated and non-inoculated plants, regardless the fertilization treatment (Table 1).

Fertilization had a significant effect on shoot-P concentration only at the first harvest (P=0.0074, Table 2), mainly due to the 15.4 % increase in P-concentration of TSP-fertilized, as compared to PR-fertilized and non-fertilized plants, respectively. At the three harvests, accumulated P in shoot tissue was also higher for inoculated plants, as compared to non-inoculated ones (P=0.0234, Table 2). P content was significantly higher in plants inoculated with PSB and fertilized with TSP than in all the other treatments (Fig. 1b).

PSB inoculation significantly affected shoot-N concentration at each of the three harvests, with P-values= 0.0054, 0.0078 and 0.0042, respectively (Table 2). Thus, N concentration of inoculated plants was 14.6, 10.0 and 13.1 % higher than non-inoculated controls for the first, second and third harvests, respectively

Table 1 The effect of P fertilization and inoculation with *P. eucalypti* M91 on the aboveground dry yield, the concentration of P and N for each harvest in shoot tissue of *L. tenuis*

Variable	P source			Inoculation		P source × Inoculation					
	-P	PR	TSP	-Inoc	+Inoc	-P-Inoc	-P+Inoc	PR-Inoc	PR+Inoc	TSP-Inoc	TSP+Inoc
Dry weight yield											
1st harvest	1837 a	1166 a	2251 a	1306 a	2197 b	1293.90 ab	2379.50 c	744.20 a	1588.50 bc	1879.00 bc	2622.40 c
2nd harvest	837 a	793 a	1229 a	904 a	1002 a	785.80 a	888.30 a	835.80 a	750.80 a	1089.30 a	1368.50 a
3rd harvest	981 a	1200 a	1461 a	1044 a	1383 a	962.30 a	999.00 a	1007.10 a	1392.30 a	1162.80 a	1758.50 a
P in shoot tissue											
1st harvest	0.13 a	0.13 a	0.15 b	0.13 a	0.15 b	0.12 a	0.13 ab	0.13 a	0.14 b	0.14 ab	0.16 c
2nd harvest	0.33 a	0.30 a	0.34 a	0.30 a	0.34 b	0.32 b	0.35 c	0.29 a	0.32 b	0.28 a	0.36 c
3rd harvest	0.21 a	0.23 a	0.22 a	0.22 a	0.23 a	0.21 a	0.22 a	0.22 a	0.24 a	0.22 a	0.22 a
N in shoot tissue											
1st harvest	1.87 a	2.04 a	2.02 a	1.84 a	2.11 b	1.84 a	1.89 ab	1.89 ab	2.19 c	1.79 a	2.25 c
2nd harvest	2.24 b	1.78 a	1.93 a	1.89 a	2.08 b	2.19 c	2.29 c	1.70 a	1.86 ab	1.76 a	2.10 bc
3rd harvest	2.20 b	2.18 b	1.98 a	1.99 a	2.25 b	2.13 b	2.28 b	2.17 b	2.19 b	1.68 a	2.28 b

Values are means of Split Plot ANOVA to analyze the effect of P-source (main factor) and inoculation with PSB (subplot factor) for each variable. Dry weight yield (kg/ha), P and N (%) for each harvest in shoot tissue of *L. tenuis*. In each line, means followed by different letters indicate significant differences ($P<0.05$) for P source and inoculation separately, and P source x inoculation according to a LSD test. –P, no fertilization; PR, fertilization with phosphate rock; TSP, fertilization with triple superphosphate; −Inoc, non-inoculated with PSB; +Inoc, inoculated with PSB. Dry weight yield at the first harvest was log transformed for this analysis and writtten back to the original data

Table 2 Split Plot ANOVA (*F* and *P* values) for the effect of P-source (main factor) and inoculation with *P. eucalypti* M91 (subplot factor) on the aboveground dry yield, the concentration of P and N, and the accumulated P and N in *L. tenuis* shoot tissue

Variable	P source		Inoculation		P source × Inoculation	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*
Dry yield						
1st harvest	3.31	0.1074	45.82	0.0001***	2.98	0.1018
2nd harvest	3.13	0.1172	0.26	0.6216	0.30	0.7512
3rd harvest	1.26	0.3481	3.38	0.0989	0.78	0.4863
Accumulated	3.71	0.0893	10.39	0.0104*	0.12	0.8916
P in shoot tissue						
1st harvest	12.40	0.0074**	21.49	0.0012**	1.37	0.3023
2nd harvest	2.94	0.1291	53.47	0.0000***	9.03	0.0071**
3rd harvest	0.52	0.6175	1.78	0.2152	0.38	0.6951
Accumulated P	2.89	0.1325	7.42	0.0234*	0.53	0.6059
N in shoot tissue						
1st harvest	0.79	0.4970	13.28	0.0054**	2.45	0.1416
2nd harvest	10.00	0.0123*	11.59	0.0078**	1.47	0.2793
3rd harvest	6.98	0.0272*	14.48	0.0042**	7.05	0.0144*
Accumulated N	2.27	0.1842	10.99	0.0090**	0.72	0.5131

F and *P* are values of Split Plot ANOVA to analyze the effect of P-source (main factor) and inoculation with PSB (subplot factor) for each variable. Dry yield (kg/ha), the concentration of P and N (%), and the accumulated P and N (kg/ha) in *L. tenuis* shoot tissue. Dry yield at the first harvest and accumulated dry yield values were log transformed for this analysis. Significant differences: *=$P< 0.05$; **=$P< 0.01$; ***=$P< 0.001$

(Table 1). Shoot-N concentration was also affected by fertilization, an effect that was evident at the second and third harvests (P=0.0123 and 0.0272, Table 2). At the second harvest, both PR and TSP fertilization exerted a negative effect on shoot-N concentration, which decreased by 20.5 % in PR fertilized plants and 13.8 % in TSP-fertilized plants, as compared to non-fertilized ones (Table 1). At the third harvest, shoot-N concentration was decreased by 10 % in TSP-fertilized as compared to non-fertilized plants, while PR-fertilized plants exerted no effect on shoot-N concentration, as compared to non-fertilized plants (Table 1).

A multiple comparison of results obtained at the first harvest showed that shoot-N concentration of inoculated plants fertilized with PR or TSP increased by 15.9 and 25.7 % respectively, as compared to non-inoculated controls (Table 1). At the second harvest, N concentration of inoculated plants increased by 19.3 % in TSP-fertilized plants, as compared to their respective non-inoculated controls (Table 1). For both non-fertilized and PR-fertilized plants, inoculation had no effect on shoot-N concentration (Table 1). At the third harvest, all the inoculated plants showed similar N concentration, were they fertilized or not, with the exception of non-inoculated plants fertilized with TSP, which exhibited lower nitrogen concentration than all the other treatments (Table 1). PSB inoculation significantly affected N accumulation in shoots at the three harvests (P=0.0090, Table 2). Accumulated N was significantly higher in plants inoculated with PSB and fertilized with TSP than in non-inoculated plants (either fertilized or non-fertilized) (Fig. 1c).

Effect of P fertilization and PSB inoculation on *L. tenuis* symbiosis with AM fungi and rhizobia

The impact of P fertilization and PSB inoculation on the symbiotic relationships between *L. tenuis* and native soil microorganisms was analyzed at the first and third harvests. Regarding the interaction with AM fungi, MC and AC indexes, as well as the number of entry points in roots were not affected by the P source and PSB inoculation at any of the two harvests (Table 3). MC and AC indexes were always high, ranging from 0.75 to 0.87 (MC) and 0.63 to 0.74 (AC) for the first harvest and from 0.64 to 0.79 (MC) and 0.54 to 0.77 (AC) for the

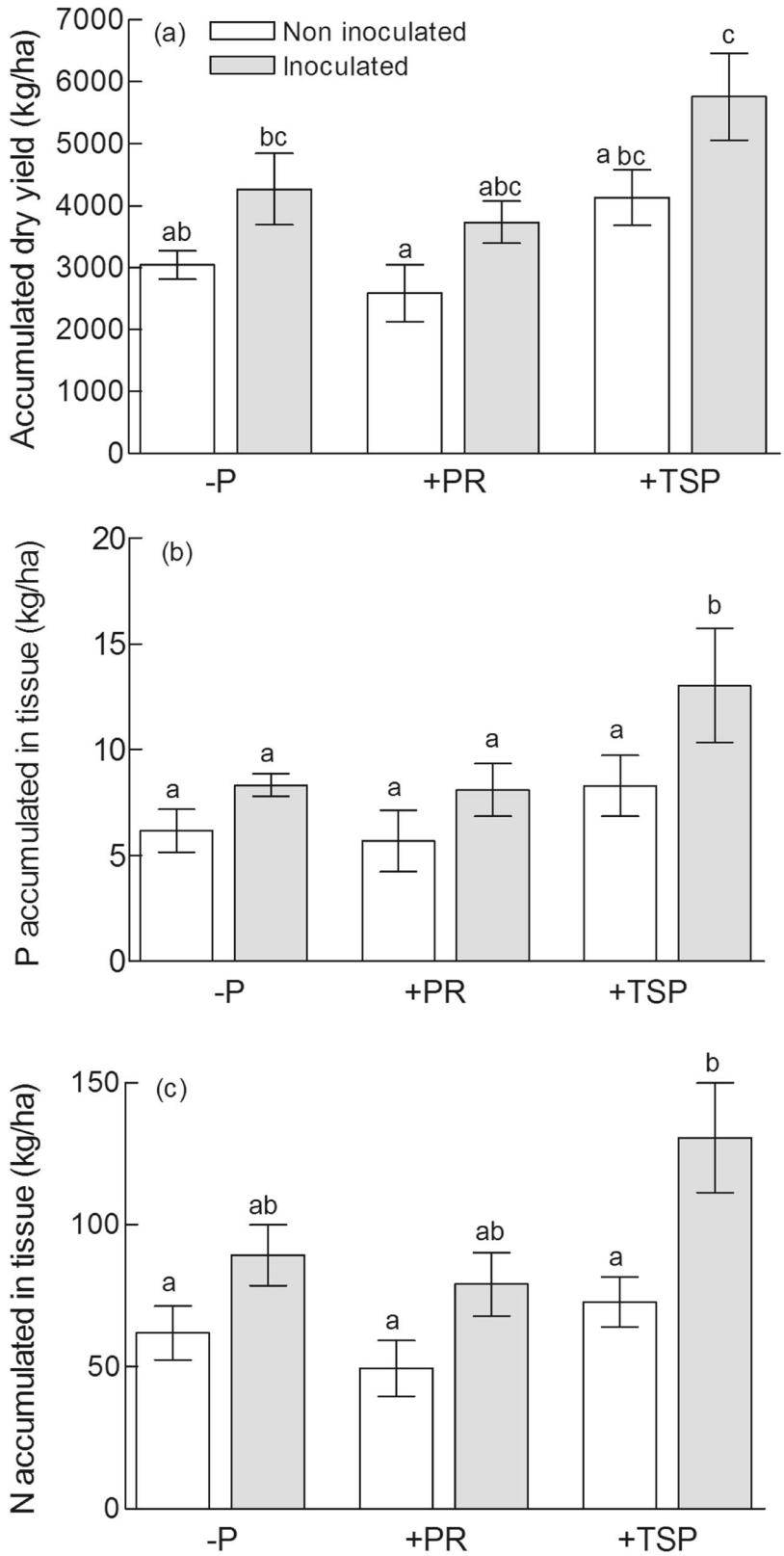

Fig. 1 Accumulated dry matter yield, P and N at the three harvests in shoot tissue of *L. tenuis* in response to P fertilization and inoculation with *P. eucalypti* M91. Accumulated dry matter, P and N, discriminated for each combination of P-source and inoculation treatment, are shown in panels (**a**), (**b**) and (**c**), respectively. Results are presented as means±standard error. Different letters indicate significant differences between treatments at a level of $P < 0.05$ by the LSD test. Accumulated dry yield was log transformed for this analysis. –P, no fertilization; +PR, fertilization with phosphate rock; +TSP, fertilization with triple superphosphate. White bars, non-inoculated plants; grey bars, PSB-inoculated plants

Table 3 Split Plot ANOVA (*F* and *P* values) for the effect of P-source (main factor) and inoculation with *P. eucalypti* M91 (subplot factor) on the performance of *L. tenuis* symbionts

Variable	P source		Inoculation		P source × Inoculation	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*
MC index						
1st harvest	0.89	0.4591	0.10	0.7552	1.13	0.3637
3rd harvest	2.04	0.2103	0.15	0.7075	1.06	0.3876
AC index						
1st harvest	0.66	0.5513	0.12	0.7323	1.11	0.3699
3rd harvest	1.22	0.3600	0.05	0.8301	1.88	0.2075
Entry points						
1st harvest	0.01	0.9934	3.77	0.0841	0.19	0.8288
3rd harvest	0.41	0.6814	0.17	0.6925	0.02	0.9789
AM spore density						
1st harvest	3.01	0.1247	21.80	0.0012**	5.35	0.0295*
3rd harvest	21.59	0.0018**	38.26	0.0002***	1.21	0.3420
Rhizobia nodules						
1st harvest	4.65	0.0592	1.81	0.2116	0.07	0.9286
3rd harvest	10.31	0.0140*	0.06	0.8196	2.45	0.1417
Available soil P						
1st harvest	5.52	0.0437*	0.26	0.6252	1.08	0.3796
3rd harvest	1.31	0.3373	1.23	0.2959	0.04	0.9650

F and *P* are values of Split Plot ANOVA to analyze the effect of P-source (main factor) and inoculation with PSB (subplot factor) for each variable. MC index: the total colonized roots of *L. tenuis* roots colonized, AC index: the fraction of root length containing arbuscules, entry points: number of entry points per mm of colonized root, Rhizobia nodules: number of rhizobia nodules per g of fresh root tissue, AM spore density: spore density in soil. Significant differences: *=$P< 0.05$; **=$P< 0.01$; ***=$P< 0.001$

third harvest. The number of entry points was 4.2 and 2.7 per mm of colonized root for the first and third harvest, respectively, and this parameter was not affected by P fertilization and PSB inoculation (Table 3).

Fertilization had no effect on AM spore density at the first harvest (P=0.1247, Table 3), while it exerted a significant effect at the third harvest (P=0.0018, Table 3). PSB inoculation affected AM spore density at both harvests (P=0.0012 and 0.0002 respectively, Table 3). At the first harvest, inoculation caused a decrease in spore density in non-fertilized and TSP-fertilized plants by 60 and 67 % respectively, but exerted no effect on spore count in soils fertilized with PR (Fig. 2a). At the third harvest, PSB inoculation increased spore density in plants exposed to all the fertilization treatments, between 33 and 73 % for PR and TSP, respectively (Fig. 2b). In this case, there was a clear negative effect of TSP fertilization on spore density both for inoculated and non-inoculated treatments (Fig. 2b).

The number of nodules per gram of root fresh weight was not affected by fertilization (P=0.0592) and PSB inoculation (P=0.2116) at the first harvest (Table 3). At the third harvest, this parameter was significantly affected by P fertilization (P=0.0140, Table 3). As opposed to fertilization, PSB inoculation had no effect on root nodulation by rhizobia (P=0.8196, Table 3). At the first harvest, the number of nodules did not differ between fertilized and inoculated plants and control plants (Fig. 2c). At the third harvest, fertilization with PR and TSP significantly reduced root nodulation of inoculated plants, as compared to non-fertilized inoculated plants (Fig. 2d).

Effect of P fertilization and PSB inoculation on soil chemical properties

Fertilization with TSP significantly increased available soil-P at the first harvest (P=0.0437, Table 3 and

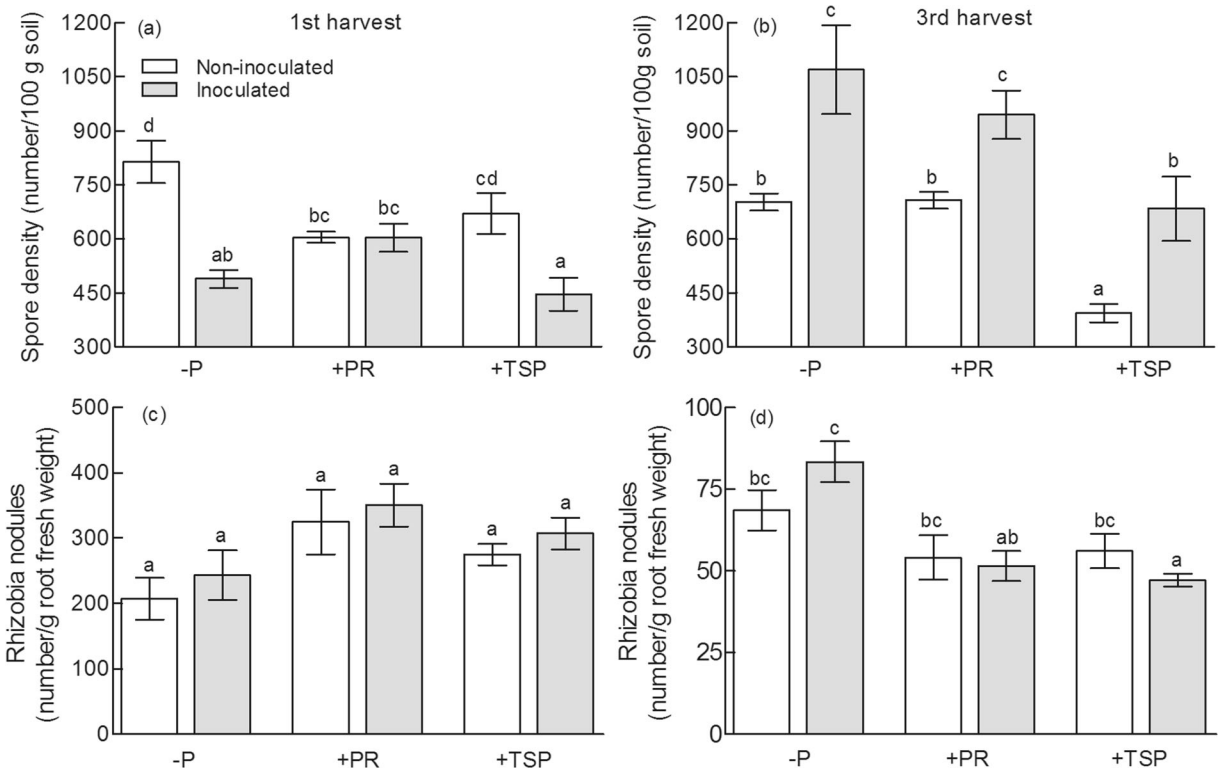

Fig. 2 Performance of *L. tenuis* symbionts in response to P fertilization and inoculation with *P. eucalypti* M91. Spore density of AM fungi in rhizospheric soil (**a** and **b**) and the number of rhizobial nodules in *L. tenuis* roots (**c** and **d**) were analyzed for the first and third harvests for each combination of P fertilization and inoculation treatment. Spore density data were arcsin transformed for this analysis and plotted back to original data. Results are presented as means±standard error. Different *letters* indicate significant differences between treatments ($P < 0.05$) according to the LSD test. –P, no fertilization; +PR, fertilization with phosphate rock; +TSP, fertilization with triple superphosphate. *White bars*, non-inoculated plants; *grey bars*, PSB-inoculated plants

Fig. 3a) but fertilization with PR had no effect on available P compared to non-fertilized soil (Fig. 3a). As opposed to the first harvest, fertilization with PR or TSP had no effect on available soil-P levels at the third harvest. In this case, available soil-P levels were similar for all treatments (Fig. 3b), being close to 10 mg P kg^{-1}

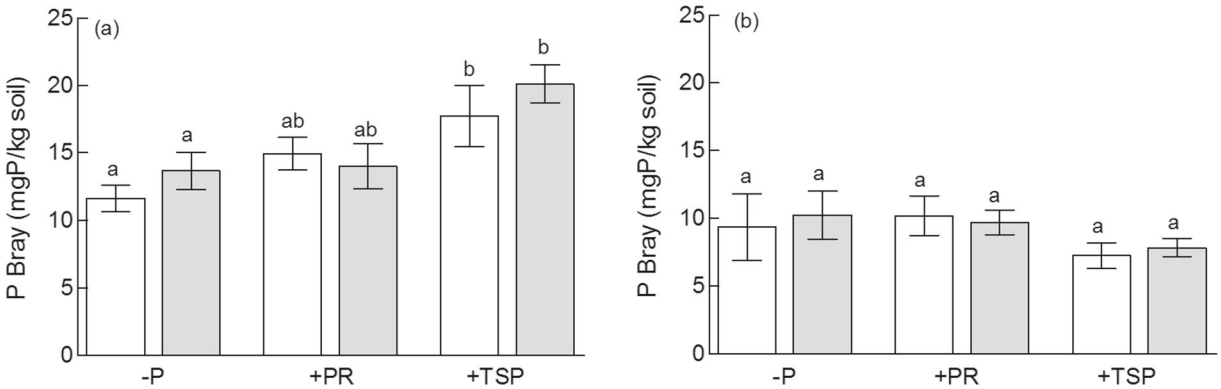

Fig. 3 Available soil-P concentration in response to P fertilization and inoculation with *P. eucalypti* M91. Available-P was analyzed in soil samples at the first (**a**) and third (**b**) harvests for each combination of P-source and inoculation treatment. Results are presented as means±standard error. Different *letters* indicate significant differences between treatments ($P < 0.05$) according to the LSD test. –P, no fertilization; +PR, fertilization with phosphate rock; +TSP, fertilization with triple superphosphate. *White bars*, non-inoculated plants; *grey bars*, PSB-inoculated plants

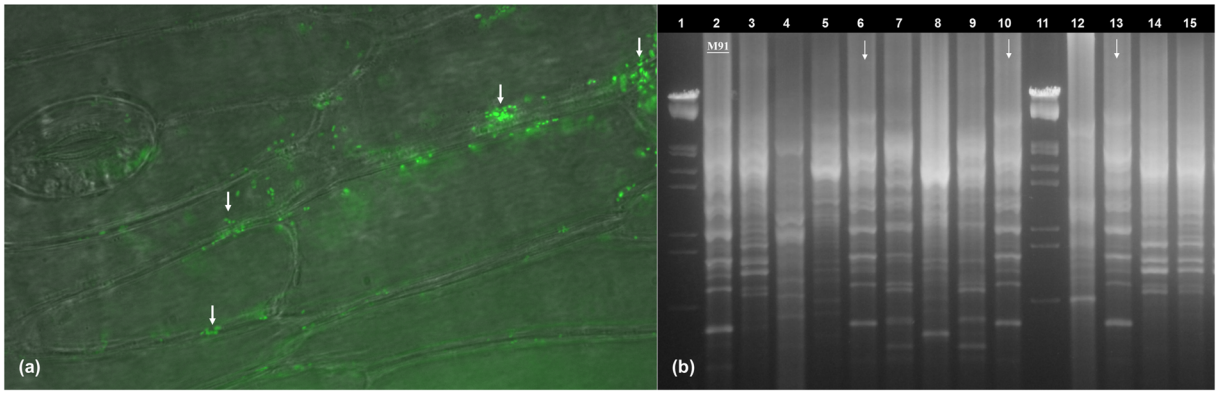

Fig. 4 Ability of *P. eucalypti* M91 to colonize *L. tenuis* plants as an endophyte. **a** Leaf-endophytic colonization of in vitro-grown plants was confirmed by acridine orange staining and epifluorescence microscopy (100×). *P. eucalypti* cells (*white arrows*) were detected in leaf intercellular spaces of inoculated plants. **b** Field-grown plants inoculated with *P. eucalypti* M91 were found by REP-PCR analysis to harbor this bacterial strain as an endophyte, both in leaves and roots. Profile analysis was carried out on a range of fragments comprised between 2027 and 125 bp, using Lambda DNA/EcoRI+HindIII as a molecular marker (lane 1 and 11). Some examples of isolates that showed REP profiles identical to strain M91 (lane 2) are indicated with *arrows*

and thus approaching to available soil-P levels measured at the first harvest for non-fertilized treatments (Fig. 3a). Inoculation with PSB had no effect on available soil-P measured by the Bray I soil test at both harvests (Fig. 3a and b). Other soil properties evaluated (pH, CE, C, N) were not significantly affected by P-fertilization or PSB-inoculation at both harvests (data not shown).

Ability of *P. eucalypti* M91 to colonize *L. tenuis* plants as an endophyte

P. eucalypti M91 was found to endophytically colonize roots and leaves of *L. tenuis* plants grown in vitro (Fig. 4a). In order to determine if this bacterial strain is also able to act as an endophyte under field conditions, phosphate-solubilizing endophytic bacteria were isolated from roots and shoots of non-fertilized plants (either inoculated or non-inoculated) at the second harvest. In this way, a total of 51 isolates were obtained (Table S1). Interestingly, PSB-inoculated plants differed from non-inoculated ones in terms of endophytic colonization, and a higher number of isolates was recovered from PSB-inoculated plants, as compared to non-inoculated controls. Moreover, no endophytic bacteria were recovered from leaves of non-inoculated plants, all the 11 isolates from these plants being derived from root samples (Table S1). On the contrary, when PSB inoculated plants were analyzed, endophytic bacteria were recovered not only from roots (24 isolates), but also from leaves (16 isolates) (Table S1).

REP-PCR profiles were obtained in order to determine if *P. eucalypti* was present within the collection of endophytic bacteria obtained from field grown plants. This analysis revealed that four out of the 24 profiles obtained from PSB-inoculated roots, as well as two out of the 16 profiles obtained from PSB-inoculated leaves were identical to that of *P. eucalypti* M91, the bacterial strain used for field inoculation (Fig. 4b). A similar analysis, based in BOX-PCR profiles, provided identical results (data not shown). Thus, the results obtained in the present work clearly demonstrate that *P. eucalypti* M91 is able to endophytically colonize *L. tenuis* plants in the field.

Discussion

The present work aimed to evaluate the potential of PSB inoculation and different P fertilizers to promote growth of *L. tenuis* plants in soils typical of the Salado River Basin. For this purpose, the native PSB *P. eucalypti* M91, previously demonstrated to promote growth of *L. tenuis plants* in vitro, was evaluated as an inoculant under field conditions, either without further amendments or in combination with chemical P-fertilizers (PR and TSP).

Previous works demonstrated that in P-deficient soils, *L. tenuis* strongly responds to the addition of this nutrient, both in monocultures and as a member of grassland communities (Ginzo et al. 1982). In the present work, *L. tenuis* was fertilized with a moderate P

dose, as compared to the higher rates (100–200 kg TSP/ha) commonly used in the region (Ginzo et al. 1982; Mendoza et al. 1984; Bailleres and Pirodi 2000). The idea behind using this moderate P dose was to minimize the risk that high levels of P fertilization masked the effects *P. eucalypti* M91 inoculation on *L. tenuis*, thus enhancing the possibility of detecting growth-promoting effects of the above mentioned bacterium.

At the first harvest, inoculation with *P. eucalypti* M91 had a positive impact on *L. tenuis* biomass production. By contrast, inoculation did not improve biomass production at the second and third harvests, as compared to non-inoculated treatments. Even though *P. eucalypti* M91 inoculation did not to promote *L. tenuis* growth during the whole experiment, the promoting effects detected at the first harvest are important due to the fact that *L. tenuis* exhibits a low growth rate during initial growth stages, which significantly decreases its ability to compete with aggressive broad leaf- or grass weeds in the grassland community (Blumenthal and McGraw 1999).

It is interesting to note that accumulated biomass production of *L. tenuis* plants was increased by inoculation with *P. eucalypti* M91 in a similar degree to TSP and PR fertilization. Thus, PSB inoculation could represent a low-cost alternative for the improvement of *L. tenuis* yield without the disadvantages associated to high rates of P fertilization (Hao et al. 2002; Gyaneshwar et al. 2002).

It's also worth to note that the application of P fertilizers (PR or TSP) without PSB had no significant effect on accumulated biomass production of *L. tenuis*. This could be the result of the moderate P concentration used in the present work to enhance the effect of PSB inoculation. Also, the drought conditions that prevailed during the experimental period, evidenced by an accumulated rainfall 35 % lower than the historical record (1911–1986), probably contributed to P immobilization after the application of fertilizers, thus leading to similar levels of available P in all the plots. In addition, the neutral and alkaline pH of the two first horizons may have contributed to decrease the solubility of fertilizers, especially PR. This situation is consistent with the values of available P found in soils of the experimental plots at the first and third harvests.

In regard to biomass production at the first harvest, PSB inoculation had a positive impact on shoot P concentration, as demonstrated by the significant increase in shoot P levels of inoculated plants, as compared to non-inoculated controls. This positive effect of PSB inoculation on shoot P concentration persisted up to the second harvest. At the first harvest, shoot P concentration was also significantly affected by fertilization. Moreover, the combination of *P. eucalypti* M91 inoculation and TSP fertilization significantly increased P accumulation in shoot tissues, as compared to other treatments. These results suggest that inoculation with *P. eucalypti* M91 increased available-P levels as a result of its solubilizing activity, thus allowing a more efficient use of P derived either from chemical fertilizers or from P compounds already present in the soil. Similar results have been reported for field trials in other crops such as rice, soybean, sunflower, maize and wheat, where an increase in biomass production and P content was observed after PSB-inoculation (Costa et al. 2013; Rana et al. 2012; Rodríguez and Fraga 1999). In a previous study by our group (Castagno et al. 2011), *P. eucalypti* M91 was found to promote *L. tenuis* growth under controlled conditions with tricalcium phosphate as the sole P source. Moreover, the abovementioned work demonstrated that the ability of this bacterial strain to solubilize tricalcium phosphate is related to gluconic and ketogluconic acid production (Castagno et al. 2011). P plant nutrition can also be improved by inoculation with bacteria that produce phytase, an enzyme capable of mineralizing organic P forms, which are highly abundant in soils (Lei et al. 2007; Pontoppidan et al. 2012). In this regard, *P. eucalypti* M91 was also found to be able to mineralize organic P forms such as Na and Ca phytate (unpublished results). On the basis of the information discussed in preceding paragraphs, the increase in P concentration and content detected in shoots of *L. tenuis* plants inoculated with *P. eucalypti* M91 could be attributed to the ability of this strain to solubilize both organic and inorganic P forms, thus increasing the availability of this nutrient in the soil.

In addition to the abovementioned effects on P nutrition, PSB inoculation also led to a significant increase in shoot-N concentration and accumulated N. On the contrary, the application of P fertilizers negatively affected shoot-N concentration, as shown by the comparison of PR- and TSP-treated plants with non-fertilized ones, particularly at the second and third harvests. Nevertheless, the highest accumulation of this nutrient in shoots was achieved by the combination of

P. eucalypti M91 inoculation and TSP fertilization, a treatment that significantly increased accumulated N in shoots as compared to non-inoculated plants, regardless of fertilization. In this regard, increased N levels in shoot tissue of legumes are often related to the establishment of a N-fixing symbiotic interaction with native soil rhizobia, or in response to the inoculation with specific rhizobial strains (Zahran 1999; Sessitsch et al. 2002). The hereby-reported increase in shoot-N concentration of *L. tenuis* plants inoculated with *P. eucalypti* M91 cannot be attributed to a stimulatory effect of *P. eucalypti* M91 on nodulation with native rhizobia, at early stages of plant growth. In this regard, nodule numbers at the first harvest showed no significant variations between different fertilization and inoculation treatments. However, *P. eucalypti* M91 was able to stimulate nodulation at later growth stages in plants cultivated in absence of P-fertilizers. This view is supported by the significant increase in the number of nodules detected in non-fertilized plants inoculated with *P. eucalypti* M91, as compared to inoculated plants fertilized with PR or TSP. Legumes are known to regulate nodulation as a function of soil N levels and plant N content (Stougaard 2000). Thus, it wouldn't be surprising that the increase in N concentration of TSP-fertilized and inoculated plants can act as a signal to down-regulate nodulation, which could in turn explain the decrease in nodule numbers detected at the third harvest in these plants

The increase in N content of plants inoculated with *P. eucalypti* M91 and fertilized with TSP, can also have resulted from an improvement of the nitrogen-fixing capacity of native rhizobia due to an increase in P availability for the energetic demands associated to nitrogenase activity. In this sense, some authors have demonstrated that biological N-fixation is highly dependent on the amount of available P in soil (Almeida et al. 2000). Therefore, part of the P solubilized by *P. eucalypti* M91 and assimilated by *L. tenuis*, could be transported to the nodules and used by the native rhizobia inside them, for metabolic processes that consume high levels of energy, (Sa and Israel 1991; Hernández et al. 2009). In addition, some *Pantoea* species are known to have nitrogenase activity and therefore increase N content in other plant species such as sugarcane (Loiret et al. 2004). Therefore, the possibility that *P. eucalypt*i M91 is able to fix atmospheric N and thus contributed to increase N-content of inoculated *L. tenuis* plants cannot be discarded.

As a summary of the beneficial effects of the combination of *P. eucalypti* inoculation and TSP fertilization discussed so far, it is important to note that even though an increase in plant biomass production was evident only at the first harvest, a positive effect on P and N accumulation was evident for longer periods, being evident up to the second and third harvest for P and N, respectively.

Symbiosis with AM fungi significantly facilitates P acquisition by plants, and their contribution to plant growth has been well-studied (Smith and Read 2008). So, the impact of P fertilization and PSB inoculation on AM fungi colonization of *L. tenuis* roots was analyzed in the present work. *L. tenuis* roots were heavily colonized by AM fungi, as previously described for plants of this species naturally grown in grasslands of the Salado River region (Escudero and Mendoza 2005; Garcia and Mendoza 2007, 2008). Mycorrhizal colonization and the number of entry points were not affected by the combination of P-fertilization and inoculation with the native phosphate-solubilizing bacterium. These results indicate that both the growth of AM fungi within the roots and the formation of new colonization units were not affected either by the P dose used or by the inoculation with *P. eucalypti* M91. It is important to highlight that biofertilization with *P. eucalypti* M91 did not affect the development of the symbiotic relationship between *L. tenuis* and the native community of AM fungi in terms of the abovementioned parameters related to root colonization. It would be interesting, however, to investigate if under controlled conditions, *P. eucalypti* M91 affects *L. tenuis*-AM fungi symbiosis from a functional point of view, as well as to analyze if both groups of soil microorganisms can develop a synergistic interaction, as previously reported for other PSB and AM fungi by several authors (Artursson et al. 2006; Muthukumar and Udaiyan 2010). Unlike intraradical colonization, spore density showed a marked increase at the third harvest, which coincided with the beginning of autumn. This increase in spore density could be attributed to a seasonal effect that became evident after the summer, a period during which the plant and fungal communities were exposed to high temperature and dry periods. Seasonality has been previously described as one of the factors able to modify the community of AM fungi in the rhizosphere of *L. tenuis* grown in different areas of the Salado River Basin (Escudero and Mendoza 2005; Garcia and Mendoza 2007, 2008). Another important point is that spore density was affected by the addition

of TSP, a highly soluble P source. Thus, the increase in P availability in soil observed at the first harvest may have inhibited the formation of new spores, as previously described by de Miranda and Harris (1994) and Smith and Read (2008).

The present work demonstrated that *P. eucalypti* M91, previously isolated from rhizospheric communities, is also able to endophytically colonize *L. tenuis* roots and leaves, both under controlled and field conditions. This finding is interesting, because the endophytic life-style could enhance the ability of this strain to survive in natural environments, once applied as an inoculant. Interestingly, under field conditions, plants inoculated with *P. eucalypti* M91 were also endophytically colonized by other PSB. In this regard, the presence of higher numbers of other endophytic PSB in *P. eucalypti* M91-inoculated plants, as compared to non-inoculated ones, suggests that inoculation with a single PSB strain somehow enhances plant colonization by other strains. The elucidation of the mechanism by which inoculation with a particular endophytic PSB leads to increased endophytic colonization by other PSB strains deserves further research. It is also worth to mention that results obtained in the present work demonstrate that specific PSB strains used as inoculants can be traced in the field, which could be a useful tool for the analysis of inoculation efficiency and the population dynamics of such bacteria in plant hosts, as well as in the environment.

The results of this study suggest that inoculation of *L. tenuis* with phosphate-solubilizing bacteria from soils of the Flooding Pampa, such as *P. eucalypti* M91 would be a sustainable option for grassland producers of this region, rather than chemical fertilizers, since it would allow a more efficient use of nutrients such as nitrogen and phosphorus.

Acknowledgments The authors wish to thank Technician Patricia Uchiya (*Comisión de Investigaciones Científicas de la Provincia de Buenos Aires*, CIC) for her valuable assistance in field trials, Ing. Agr. María Cristina Pomar for determining nutrient determination in plant tissue and Dr. Fernando Pieckenstain for helpful discussion.

This study was supported by: *Comisión de Investigaciones Científicas de la Provincia de Buenos Aires* (CIC, Argentina); *Consejo Nacional de Investigaciones Científicas y Técnicas* (CONICET, Argentina) and *Agencia Nacional de Promoción Científica y Tecnológica* (Argentina, PICT). LNC is a post-doctoral fellow of CONICET. IVG, AIS, REM and OAR are researchers of CONICET. MJE is a member of the Research Career of CIC.

References

Almeida JF, Hartwig UA, Frehner M, Nösberger J, Lüscher A (2000) Evidence that P deficiency induces N feedback regulation of symbiotic N_2 fixation in white clover (*Trifolium repens* L.). J Exp Bot 51:1289–1297

Amijee F, Tinker PB, Stribley DP (1989) The development of endomycorrhizal root systems. VII. A detailed study of effects of soil phosphorus on colonization. New Phytol 111: 435–446

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10

Bailleres M, Pirodi F (2000) Fertilización a campo natural en la chacra experimental Manantiales. Ida Vuelta Rural (INTA) 11:5–6

Blumenthal MJ, McGraw RL (1999) *Lotus* adaptation, use and management. In: Beuselinck PR (ed) Trefoil: the science and technology of *Lotus*. American Society of Agronomy Inc. & Crop Science Society of America Inc, Wisconsin, pp 97–119

Bremmer JM, Mulvaney CS (1982) Nitrogen total. In: Black CA (ed) Methods in soil analysis: agronomy. American Society of Agronomy. Inc. Madison, Wisconsin, pp 595–624

Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA (2011) Phosphate-solubilization mechanism and in vitro plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina). J Appl Microbiol 110:1151–1165

Costa P, Beneduzi A, Souza R, Schoenfeld R, Vargas L, Passaglia LP (2013) The effects of different fertilization conditions on bacterial plant growth promoting traits: guidelines for directed bacterial prospection and testing. Plant Soil 368:267–280

Daniels N, Skipper H (1982) Methods for the recovery and cuantitative estimation of propagules from soil. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathology Society, St. Paul, pp 29–35

De Miranda JCC, Harris PJ (1994) Effects of soil phosphorus on spore germination and hyphal growth of arbuscular mycorrhizal fungi. New Phytol 128:103–108

Díaz P, Borsani O, Monza J (2005) *Lotus*-related species and their agronomic importance. In: Márquez JS AJ, Udvardi M, Parniske M, Spaink H, Saalbach G, Webb J, Chiurazzi M (eds) *Lotus* japonicus Handbook. Springer, Netherlands, pp 25–37

Escudero V, Mendoza R (2005) Seasonal variation of arbuscular mycorrhizal fungi in temperate grasslands along a wide hydrologic gradient. Mycorrhiza 15:291–299

Garcia IV, Mendoza RE (2007) Arbuscular mycorrhizal fungi and plant symbiosis in a saline-sodic soil. Mycorrhiza 17:167–174

Garcia IV, Mendoza RE (2008) Relationships among soil properties, plant nutrition and arbuscular mycorrhizal fungi-plant symbioses in a temperate grassland along hydrologic, saline and sodic gradients. FEMS Microbiol Ecol 63:359–371

Ginzo HD, Collantes M, Caso OH (1982) Fertilization of a native grassland in the "Depresión del Río Salado", Province of Buenos Aires: Herbage dry matter accumulation and botanical composition. J Range Manage 35:35–39

Gyaneshwar P, Naresh Kumar G, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245:83–93

Hao X, Cho CM, Racz GJ, Chang C (2002) Chemical retardation of phosphate diffusion in an acid soil as affected by liming. Nutr Cycl Agroecosys 64:213–224

Hernández G, Valdés-López O, Ramírez M, Goffard N, Weiller G, Aparicio-Fabre R, Fuentes SI, Erban A, Kopka J, Udvardi MK, Vance CP (2009) Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. Plant Physiol 151:1221–1238

Igual JM, Valverde A, Cervantes E, Velázquez E (2001) Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. Agronomie 21:561–568

Jackson ML (1964) Soil chemical analysis. Prentice Hall, Englewood Cliffs

Kurtz LT, Bray RH (1945) Determination of total, organic and available forms of phosphorus in soils. Soil Sci 59:39–45

Lei XG, Porres JM, Mullaney EJ, Brinch-Pedersen H (2007) Phytase: source, structure and application. In: Polaina J, MacCabe AP (eds) Industrial enzymes. Springer, Netherlands, pp 505–529

Loiret FG, Ortega E, Kleiner D, Ortega-Rodés P, Rodés R, Dong Z (2004) A putative new endophytic nitrogen-fixing bacterium *Pantoea* sp. from sugarcane. J Appl Microbiol 97:504–511

Mc Gonigle TP, Miller MH, Evans DG, Fairchaild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

Mendoza RE, Cogliatti DH, Collantes MB, Kade M (1984) Efecto de la fertilización nitrógeno-fosfatada sobre el crecimiento otoño-invernal y la absorción de fósforo en tepes de un pastizal natural. Turrialba 33:311–320

Monier JM, Lindow SE (2004) Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. Appl Environ Microbiol 70:346–355

Montes L (1988) *Lotus tenuis*. Rev Argent Prod Anim 8:367–376

Muthukumar T, Udaiyan K (2010) Growth response and nutrient utilization of *Casuarina equisetifolia* seedlings inoculated with bioinoculants under tropical nursery conditions. New For 40:101–118

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Phillips JM, Hayman DS (1970) Improved procedure for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Pontoppidan K, Glitsoe V, Guggenbuhl P, Quintana AP, Nunes CS, Pettersson D, Sandberg A-S (2012) In vitro and in vivo degradation of myo-inositol hexakisphosphate by a phytase from *Citrobacter braakii*. Arch Tierernahr 66: 431–444

Rana A, Joshi M, Prasanna R, Shivay YS, Nain L (2012) Biofortification of wheat through inoculation of plant growth promoting rhizobacteria and cyanobacteria. Eur J Soil Biol 50:118–126

Richter M, Wistinghausen EV (1981) Unterscheidbarkeit von humusfraktione in boden 9 bei unterscheidlicher bewirtschaftung. Z Pflanzenernaeht Bodenk 144:395–406

Robson AD, O'Hara GW, Abbott LK (1981) Involvement of phosphorus in nitrogen 11 fixation by subterranean clover (Trifolium subterraneum L.). Aust J Plant Physiol 8:427–436

Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17: 319–339

Sa T-M, Israel DW (1991) Energy status and functioning of phosphorus-deficient soybean nodules. Plant Physiol 97: 928–935

Sannazzaro AI, Bergottini VM, Paz RC, Castagno LN, Menendez AB, Ruiz OA, Pieckenstain FL, Estrella MJ (2011) Comparative symbiotic performance of native rhizobia of the Flooding Pampa and strains currently used for inoculating *Lotus tenuis* in this region. Anton Leeuw Int J G 99:371–379

Sessitsch A, Howieson JG, Perret X, Antoun H, Martínez-Romero E (2002) Advances in *Rhizobium* Research. Crit Rev Plant Sci 21:323–378

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London, p 815

Sperry JF, Wilkins TD (1976) Arginine, a growth-limiting factor for *Eubacterium lentum*. J Bacteriol 127:780–784

Stougaard J (2000) Regulators and regulation of legume root nodule development. Plant Physiol 124:531–540

Taurian T, Anzuay MS, Angelini JG, Tonelli ML, Ludueña L, Pena D, Ibáñez F, Fabra A (2010) Phosphate-solubilizing peanut associated bacteria: Screening for plant growth-promoting activities. Plant Soil 329:421–431

Vyas P, Gulati A (2009) Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC Microbiol 9:174

Wermbter R, Ramallo D (1980) Carta detallada de suelos de la Chacra Experimental Mananatiales. Departamento de suelos. INTA, pp39-42

Zahran HH (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol R 63:968–989