Prospecting phosphate solubilizing bacteria in alkaline-sodic environments reveals intra-specific variability in *Pantoea eucalypti* affecting nutrient acquisition and rhizobial nodulation in *Lotus tenuis*

Liz Marjory Cumpa-Velásquez, Jorge Ignacio Moriconi, Diana Patricia Dip, Luis Nazareno Castagno, María Lucrecia Puig, Santiago Javier Maiale, Guillermo Esteban Santa-María, Analía Inés Sannazzaro, María Julia Estrella [*]

Instituto Tecnológico Chascomús (INTECH), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), - Universidad Nacional de San Martín (UNSAM), Avenida Intendente Marino, Km 8.2, (7130) Chascomús, Buenos Aires, Argentina

ABSTRACT

A bioprospecting study in alkaline-sodic soils of the Argentinean flooding pampa was performed in order to identify and characterize rhizospheric bacteria associated to *Lotus tenuis* plants, capable of solubilizing phosphate under a broad range of alkaline-sodic conditions. Our analysis, supported by repetitive BOX element based PCR and 16S rRNA sequences, identified 74 strains. All of them belong to the Phylum *Proteobacteria*, specifically to the order *Enterobacteriales,* and *Pseudomonadales*, suggesting that in this environment, broad pH-range P-solubilizing bacteria (BRPSB) associated to *L. tenuis*, are grouped within a narrow taxonomic range. A subsequent objective was to focus in a subgroup of BRPSB strains belonging to the *Pantoea eucalypti* species (MA66, P63, P76, P163, P173 and a formerly identified isolate, M91) that also produced siderophores, indol-acetic acid and showed *in vitro* compatibility with the native rhizobial strain *Mesorhizobium sanjuanii* BSA136. Growth promoting effects of these *P. eucalypti* strains on *L. tenuis* plants in alkaline-sodic soils in symbiosis with the above mentioned rhizobial strain were analyzed. Despite all the *P. eucalypti* BRPSB strains exhibited the above-mentioned features, they exerted differential effects on plant growth and dry matter allocation to the nodules. Plants inoculated with *P. eucalypti* M91 displayed a superior capability to accumulate nitrogen, phosphorus and zinc. On the contrary, nodules dry matter allocation, and mineral nutrient accumulation in *L. tenuis* plants were negatively affected by *P. eucalypti* P76 compared with M91. Results hereby presented highlight the complexity of plant-microbe interactions and reveal that growth-promoting effects of P-solubilizing *P. eucalypti* strains cannot be predicted only on the basis of their *in vitro* PGPR features, complementary *in planta* assays being necessary for efficient strain selection. This study provides valuable information for biofertilization of *L. tenuis* plants in the flooding pampa.

1. Introduction

Salinization and sodification are important drivers of soil degradation that significantly affect crop production (Eynard et al., 2005; Pla-Sentís, 2014). Particularly, agricultural production is affected in alkaline-sodic soils by the deterioration of soil structure and permeability, which restrict the movement of water to the root zone and affect seedling emergence, thus hampering plant development. These soils contain appreciable amounts of sodic salts that, undergoing alkaline

hydrolysis, increase the pH of the soil solution. Additionally, alkaline conditions affect the availability of some macro and micronutrients required by plants (Adcock et al., 2007). In particular, phosphorus (P) availability is severely affected in alkaline soils, mainly as a result of its high rate of fixation into insoluble complexes with calcium (Hopkins and Ellsworth, 2005). Therefore, plants dwelling in alkaline soils should be able to cope with a plethora of simultaneous stress conditions that compromise plant productivity.

Plant Growth Promoting Bacteria (PGPB) may provide a reasonable

and sustainable approach to counteract the adverse effects of alkalinity and sodicity on plant growth and development given the genetic and metabolic diversity of the microbiota associated with roots and the beneficial effect of some of its members on plant performance (Dixit et al., 2020; Torbaghan et al., 2017). Phosphate solubilizing bacteria (PSB), a subgroup of PGPB with the capability to increase P availability, play a key role in low P-availability soils. This capability rests on different mechanisms, including the release of various compounds (mainly organic acids) that solubilize P (Alori et al., 2017; Sharma et al., 2013). Several bacterial genera bearing this ability have been described, but just few of them (such as *Agrobacterium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Paenobacillus, Pantoea, Pseudomonas, Serratia* and *Streptomyces*) are currently used for commercial microbial formulations (Glick, 2012). Noticeably, when selected strains are applied in diverse environments, highly variable outcomes can be observed in relation to their effects on the promotion of plant growth (Owen et al., 2015), probably as a consequence of competition with native strains that are potentially better adapted to local soil conditions. In this regard, several studies have indicated that the detection, selection, and application of rhizobacteria isolated from adverse environments with enhanced capacity to tolerate specific stress conditions, may be a suitable strategy to mitigate the detrimental effects suffered by crops in such environments (Etesami and Beattie, 2018).

Salt-tolerant PGPB native from saline soils, were found to alleviate the negative effects of salinity on plants (He et al., 2018; Sharma et al., 2016). For alkaline-sodic soils the literature is still scarce, but recent studies suggest that the use of alkali-tolerant PGPB can enhance wheat, alfalfa and corn productivity under alkaline conditions (Dixit et al., 2020; Liu et al., 2019; Torbaghan et al., 2017). In this sense, bioprospecting of rhizobacteria associated with valuable plant species growing in alkaline-sodic soils is of great interest to reveal the microbial diversity of unexplored restrictive environments; thus allowing selecting novel microorganisms that act as nutrient suppliers for plant growth in the abovementioned environments. An important criterion for selecting such microorganisms is their ability to growth over a wide range of soil conditions, including soil pH, which is a major driver of the structure of soil bacterial communities (Zheng et al., 2019).

In Argentina, the flooding pampa is a large area dedicated to cattle raising (Rubio et al., 2019), one of the country's valuable export products. The ongoing expansion of agriculture has led to a reduction of the surface devoted to cattle raising, which has been shifted to marginal soils, particularly the alkaline-sodic/saline lowlands. *Lotus tenuis*, a Fabaceae originary of Eurasia, has long been considered the legume species of choice for cattle feeding given its forage quality and its performance in restrictive environments that include a wide soil pH range (Rubio et al., 2019; Stoffella et al., 1998; Teakle et al., 2010). The productivity of legumes of the *Lotus* genus is strongly influenced by the symbiosis with nitrogen-fixing-bacteria (Lorite et al., 2018; Sannazzaro et al., 2011). In a previous search for rhizospheric PSB associated to naturalized *L. tenuis* plants in soils of the flooding pampa covering a pH range of 6.0 to 8.0 (Castagno et al., 2011) we selected a *Pantoea eucalypti* strain that significantly improved shoot dry matter production and P nutrition when inoculated on plants grown in neutral soils (Castagno et al., 2014). However, the diversity of PSB of the most constrained environments of the flooding pampa (*i.e.* the alkaline-sodic lowlands) has not been explored yet. Importantly, a wide variation of soil pH can be found in the flooding pampas, with *L. tenuis* dwelling over such a broad pH range (Stoffella et al., 1998). Therefore, we thought it would be interesting to identify bacteria able to solubilize P at neutral pH as well as under alkaline-sodic conditions. Furthermore, the impact of these native microorganisms on nitrogen-fixing rhizobia, as well as on plant growth and mineral composition, has yet to be explored.

We aimed to test the hypothesis that the rhizosphere of *Lotus tenuis* plants growing in alkaline-sodic soils of the flooding pampa harbour an important genetic and taxonomic diversity of PSB with the capability to grow at neutral pH as well as under alkaline conditions in the presence of sodium. One of the groups identified in this work contained several strains related to *Pantoea eucalypti*. We hypothesized that even when these closely related strains may share similar *in vitro* attributes, their effect on *L. tenuis* plants would need further evaluation. The previously reported potential of this bacterial species for biofertilization of *L. tenuis* plants in neutral soils of the flooding pampa prompted us to further characterize this group. Thus, a complementary objective was to characterize a subset of *P. eucalypti* strains and evaluate their impact on growth and mineral composition of *Lotus tenuis* in co-inoculation trials with a nitrogen-fixing rhizobial strain native from these soils.

2. Materials and methods

2.1. Sampling sites for the isolation of phosphate solubilizing bacteria (PSB)

During June 2015, rhizospheric soil samples were obtained from *Lotus tenuis* plants growing in typical Natracualf soils, locally named as alkaline-sodic lowlands, from Manantiales (35°44′36.319″S -58°3′25.307″W) and Punta Indio (35°16′ 15.449″ S -57°14′ 52.101″ W), both located in the flooding pampa region. Eight and nine plants were sampled in each site, respectively. Sampling was carried out in plots of approximately 50 × 50 m, and the plants collected were spaced approximately 10 m from each other. Samples were stored at 4 °C during 24 h, until bacterial isolation.

2.2. Soil analysis

The soil samples (top 0–20 cm) collected were analyzed following protocols standardized by the Argentinean Network of Agricultural Laboratories (REDLAA). Particularly, the electric conductivity (EC) and pH were determined in a soil/water suspension (1:2.5; w/v) using a Hanna HI255 pH and conductivity meter, with a Hanna HI1332 pH electrode and a Hanna HI76310 conductivity electrode. Cationic Exchange Capacity (CEC) was quantified by the ammonium acetate method (Sparks et al., 1996). The Oxidizable Organic Carbon (C) and Organic Matter (OM) were measured by the Walkley-Black method (Walkley and Black, 1934). Calcium (Ca^{2+}) and magnesium (Mg^{2+}) contents were determined by complexometric titration and sodium (Na^+) and potassium (K^+) concentrations were measured by flame photometry. Soil nitrogen (N) and phosphorus (P) were determined by the Kjeldahl (Bradstreet, 1954) and Bray-Kurtz (Bray and Kurtz, 1945) methods, respectively.

2.3. Isolation of rhizospheric PSB

PSB were isolated from rhizospheric soil collected from individual plants. Soil particles tightly adhered to plants roots were removed by washing and agitation with a sterile 10 mM $MgCl_2$ solution. Serial dilutions of rhizospheric soil were plated on agarized NBRIP medium (Nautiyal, 1999) with tricalcium phosphate as the sole P source. Plates were incubated at 28 °C for 7 days. Colonies surrounded by a clear halo were considered positive for phosphate solubilization ability and this phenotype was confirmed by repeatedly sub-culturing on NBRIP medium. Phosphate solubilizing isolates were grown in liquid Tryptone-Yeast (TY) medium (Sperry and Wilkins, 1976) and preserved with glycerol 30% at −80 °C.

2.4. Assessment of phosphate solubilizing ability under neutral and alkaline-sodic conditions

Bacteria were grown in liquid TY medium on a rotary shaker at 28 °C until exponential growth phase (24 h), pelleted by centrifugation, washed twice and resuspended in 10 mM $MgCl_2$ solution. The ability to solubilize phosphate under alkaline-sodic conditions was evaluated in NBRIP medium with tricalcium phosphate, with pH values adjusted to

8.0, 9.0 and 10.0 (solid medium) or 8.0 and 9.0 (liquid medium) with Na_2CO_3. NaCl was added to a final concentration of 200 mM Na^+. NBRIP at pH 7.0 without Na^+ addition was used as a control. For the determination of phosphate solubilizing activity in solid medium, 10 μL aliquots of bacterial suspensions containing 1×10^6 colony forming units (CFU) mL^{-1} were inoculated by duplicate on agarized NBRIP medium and incubated at 28 °C for 168 h. A phosphate solubilizing index (PSI) was calculated as: PSI = A/B where A is the diameter of the colony plus the cleared area around it and B is the colony diameter (Sarkar et al., 2012). For liquid medium assays modified NBRIP (10 mL) was inoculated with bacterial suspensions (1×10^5 CFU mL^{-1}) and incubated in a rotary shaker at 28 °C. Samples were collected 24, 72 and 168 h post-incubation (hpi) and centrifuged at 14.000 rpm for 5 min. Soluble P (molybdenum-blue method) and pH were determined in the supernatants thus obtained.

2.5. Siderophore production under alkaline-sodic conditions

Bacterial isolates were grown as described in section 2.4, then 10 μL aliquots were spotted on solid TY (pH 9.0) amended with 200 mM Na^+. TY (pH 7.0) without NaCl addition was used as a control. After incubation at 28 °C for 24 h, Chrome Azurol S (CAS) agarized medium (Pérez-Miranda et al., 2007) was overlaid on the growing cultures and further incubated. Siderophores production was evidenced by the formation of an orange to yellow halo around the colonies.

2.6. Colorimetric estimation of Indol-3-acetic acid

To assess the ability to produce compounds with auxin activity, isolates were grown overnight in liquid TY medium supplemented with 10 μM L-tryptophan (Sigma-Aldrich, USA) on a rotary shaker at 28 °C. Triplicate samples (100 μL) were obtained from each strain 72 hpi, and indole-3-acetic acid (IAA) was determined. Briefly, after centrifugation for 5 min at 6.000 rpm, supernatants were mixed with 100 μL Salkovski reagent and incubated for 30 min in the dark. IAA production was estimated by determining absorbance at 530 nm in a microplate reader (Synergy H1, BioTek, USA). IAA concentration was calculated using a calibration curve with commercial IAA (Sigma-Aldrich, USA).

2.7. BOX-PCR fingerprinting

PSB were grown on TY-agar for 24 h at 28 °C, then a loopful of biomass from each isolate was suspended in 20 μL of lysis solution (0.01 M NaOH +0.25% SDS) and boiled at 100 °C for 30 min. After addition of 0.1 mL of sterile milliQ water, cell suspensions were centrifuged at 13.500 g for 2 min and the supernatant was used as source of genomic DNA for BOX-PCR amplification reactions using the universal BOXAIR1 primer synthesized by Invitrogen, Argentina (Sannazzaro et al., 2011). PCR products together with a 1Kb Plus Ladder DNA marker (Fermentas) were separated on a 1.5% agarose gel stained with ethidium bromide. Each BOX-PCR fingerprinting was analyzed through the Bionumerics software (Applied Maths, Belgium, temporary Bionumerics evaluation license). Similarity matrices were calculated using the Pearson's product moment correlation coefficient followed by a dendrogram construction using the unweighted pair group method with the arithmetic averages (UPGMA) algorithm (Sneath and Sokal, 1973). Cluster analysis of BOX fingerprints patterns was done at 75% similarity.

2.8. Phylogenetic analysis of 16SrRNA, gyrB and rpoB housekeeping genes

Total genomic DNA was extracted from individual isolates using the AccuPrep Genomic DNA Extraction Kit (*Bionner*, USA), according to the manufacturer's instructions. Nearly full-length 16S rRNA gene was amplified for each isolate using the universal primers 41f and 1488r as described by Estrella et al. (2009). Fragments of *gyr*B and *rpo*B genes

were amplified for *P. eucalypti* strains, using 320F/1260R and rpoBCM7-F/rpoBCM31b-R primers, respectively (Dauga, 2002; Brady et al., 2008). Both strands of the resulting amplicons were sequenced (Macrogen, Korea). To establish the phylogenetic position of the strains individual and concatenated gene sequences were aligned with reference sequences, obtained from The Ribosomal Database Project Release 11.5 (Cole et al., 2014) and the National Center for Biotechnology Information (NCBI) Genbank Database (https://www.ncbi.nlm.nih.gov/), using the Clustal module implemented by the MEGA software version 7.0 (Kumar et al., 2018). Distances were calculated using a complete-deletion procedure and the maximum-likelihood method was used to construct phylogenetic trees. The robustness of tree topologies was evaluated by bootstrap analysis (1.000 replicates). Nucleotide sequences of 16S rRNA, *gyr*B and *rpo*B genes were deposited in GenBank (Supplementary Tables S3 and S4). Accession numbers of the nucleotide sequences of 16S rRNA, *gyr*B and *rpo*B genes of the reference strains are provided in supplementary Tables S5 and S6.

2.9. Compatibility test between isolated strains and rhizobia

PSB selected for an in planta experiment (see below) were tested for their compatibility with a rhizobial strain by the cross streak method. Each PSB strain was streaked across the surface of a TY or TY modified (pH 8.0, 150 mM Na+) agar plate and the rhizobia strain was cross-streaked perpendicularly. Plates were incubated for 5 days at 28 °C. The presence of inhibition zones at the intersection of the tested isolates was next analyzed.

2.10. Effect of phosphate solubilizing bacteria on Lotus tenuis growth, nutrition and symbiotic rhizobial interaction

Five phosphate solubilizing *Pantoea eucalypti* strains isolated in this study, as well as the previously described M91 strain (Castagno et al., 2011), were individually co-inoculated on *L. tenuis* plants with *Mesorhizobium sanjuanii* BSA136, a native nitrogen fixing rhizobia formerly isolated from nodules of *L. tenuis* plants grown in alkaline-sodic soils of the area under study (Estrella et al., 2009; Sannazzaro et al., 2018). Bacteria were grown at 28 °C in liquid TY medium for 24-48 h (PSB) or 72 h (M. sanjuanii BSA136) up to the exponential growth phase. Cells were harvested and washed twice by centrifugation with sterile 10 mM $MgCl_2$. Inoculum was prepared by re-suspending bacterial pellets in sterile 10 mM $MgCl_2$ to a density of 10^8 cells mL^{-1}.

L. tenuis cv. Bartriunfo, which displays high tolerance to salinity and sodicity conditions and is widely cultivated in the flooding pampas, was used for this experiment. Seeds, kindly provided by Barenbrug (Pergamino, Argentina) were surface disinfected and allowed to germinate in the dark for 72 h at 25 °C (Estrella et al., 2009). Following germination, two seedlings were transferred to plastic pots containing 300 mL of a sterilized soil-sand mixture (4:1 V/V) and each seedling was inoculated with 1 mL of a suspension containing 10^8 cells of each bacterial strain.

An alkaline sodic soil from the flooding pampa (Chascomús, 35° 38′001.1″S -58° 00′07.7″W) was used in this experiment mixed with sand (4:1 V/V). Soil physicochemical properties were: pH: 8.2, EC: 0.8 ds/m; C: 2.24%; OM: 4.48%; P: 7.90 ppm; Nt: 0.23% C/N ratio: 9.74%; Ca^{2+}: 1.12 meq/100 g; Mg^{2+}:0.49 meq/100 g; Na^+: 1.22 meq/100 g; K^+: 0.15 meq/100 g and CEC: 3.18 meq/100 g. It was checked that EC and pH of the soil:sand mix were the same as in the original soil. Plants were grown in a controlled environment under 16/8 h ligth/dark cycle at 25/21 °C (day/nigth). The photon flux density at the plant level was 200 μmol m^{-2} s^{-1}, provided by incandescent and cool white fluorescent lamps. Once field capacity was achieved, pots were irrigated from the bottom every two days using sterile distilled-water. A completely randomized design was used, with two plants per pot and eight pots per treatment. Each treatment consisted in the co-inoculation of *L. tenuis* seedlings with each selected PSB and *M. sanjuanii* BSA136, except for controls, which were inoculated only with the rhizobial strain.

Plants were harvested 40 days after inoculation. Shoot and root fresh weights were determined and nodules carefully separated from roots. Shoots, roots and nodules were dried at 60 °C until constant weight was reached. Root and shoot nitrogen content was estimated by micro-Kjedhal method. For the rest of the determinations, 100 mg of dry material was reduced to ashes at 550 °C and the residues were suspended in 3.5 mL of a 65% HNO_3:H_2O (1:6 V/V) solution. The concentration of Potassium (K), Calcium (Ca), Magnesium (Mg), Phosphorus (P), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn) and Sodium (Na) in the digests was estimated by microwave plasma atomic emission spectroscopy (MP-AES 4200, Agilent).

2.11. Statistical analysis

For all variables the homogeneity of variance and normality were assessed by using diagnostic plots (Di Rienzo et al., 2008). When departure from homogeneity of residues and/or normality was detected, data transformation procedures (Log, Box-Cox, Inverse Normal Transformation) were used to improve them. Analysis of variance (ANOVA) was achieved using the Infostat software (Di Rienzo et al., 2008). Factorial ANOVA was performed for the solubilization assay in liquid media, while One Way ANOVA was performed for the remaining analyses, as indicated in the figure legends. Post-hoc comparisons were carried out with the Tukey's multiple-comparison test.

3. Results

3.1. Isolation and selection of bacteria able to solubilize phosphate under alkaline-sodic conditions

Two sites in the flooding pampa with an exchangeable sodium percentage (ESP) higher than 15 and pH higher than 8.5 (Supplementary Table S1) were sampled in order to isolate, and subsequently identify, phosphate solubilizing bacteria from the rhizospheric microbiota of *Lotus tenuis* plants naturally growing under alkaline-sodic conditions. Following the screening for the formation of cleared zones around bacterial colonies grown on pH 7.0 NBRIP medium, a total of 310 P-solubilizing isolates were obtained from both locations. Next, each isolate was tested for its P-solubilization ability at different external pH conditions on NBRIP agar modified. Out of a total of 310 PSB isolates, 299 proved to be able to solubilize phosphate under alkaline-sodic conditions, representing 96.45%, while the remaining 3.55% only formed halos at pH 7.0. The isolates that formed haloes were classified as narrow (pH 7.0–8.0 + Na+), intermediate (pH 7.0–9.0 + Na+) and broad (pH 7.0–10.0 + Na+) range P solubilizing bacteria (NRPSB, IRPSB and BRPSB, respectively). Based on the aforementioned classification, 35 isolates (11.70%) were classified as NRPSB, 190 (63.55%) as IRPSB, and 74 (24.75%) as BRPSB (Supplemental Table S2). As this work was aimed to identify bacteria able to solubilize P at neutral pH as well as under alkaline conditions in the presence of sodium, BRPSB isolates were selected for further genetic and taxonomic characterization.

3.2. Genetic and taxonomic diversity of BRPSB

The genetic diversity of the 74 BRPSB was evaluated by analyzing genomic DNA fingerprints obtained by the BOX-PCR method. Through UPGMA-based cluster analysis with a 75% similarity cut-off level, the isolates were classified into 36 clusters (I to XXXVI), 21 of them consisting in a single isolate and the remaining 15 ranging from 2 to 8 isolates (Fig. 1, Table 1). To estimate the taxonomical diversity among isolates of different clusters, PCR amplification followed by analysis of 16S rRNA sequences was performed for one isolate of each cluster. The 16S rRNA sequences of these 36 isolates were then compared with the sequences included in the Ribosomal Database Project in order to identify bacterial taxa closely related to our BRPSB. This analysis revealed that the majority of the isolates (33) were related to different
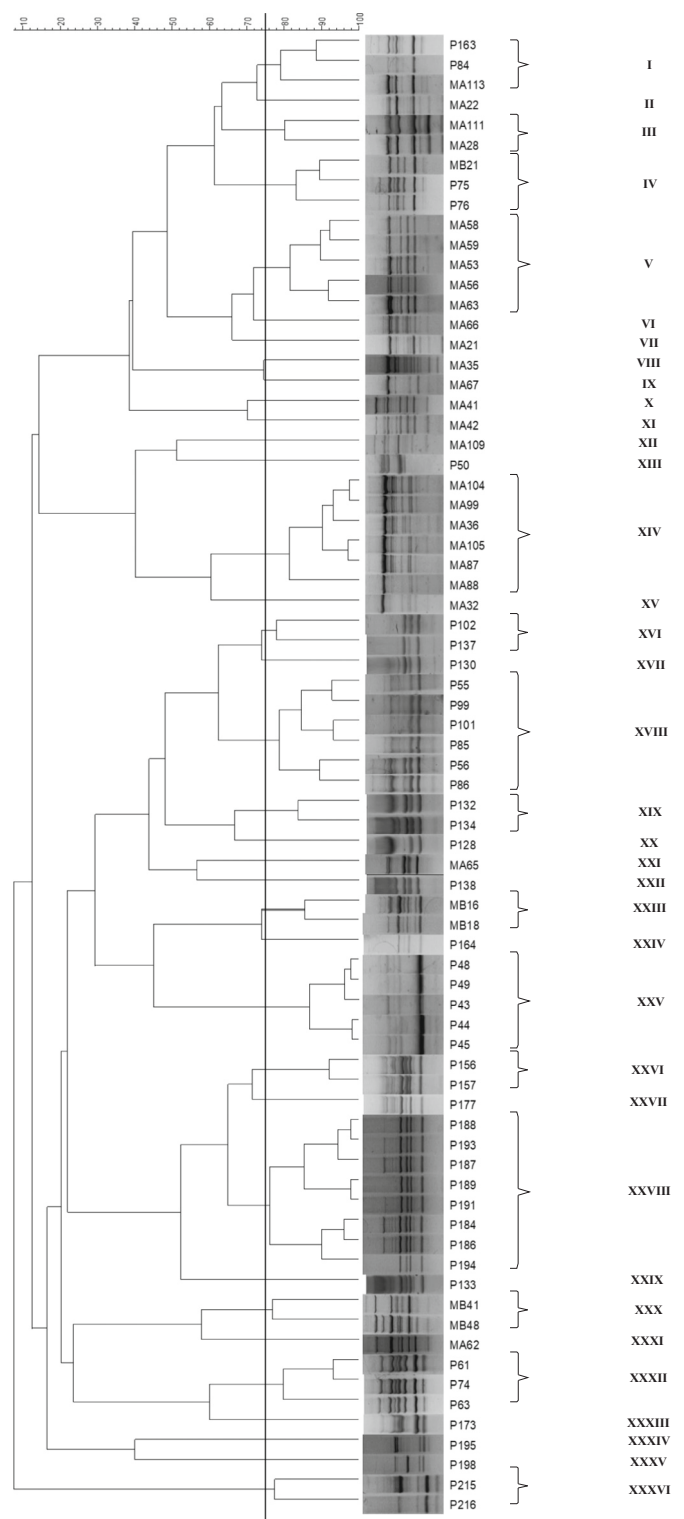

Fig. 1. UPGMA similarity tree of BOX-PCR patterns of Broad Range Phosphate-Solubilizing Bacteria (BRPSB) isolated from the rhizosphere of *Lotus tenuis* plants naturally grown in alkaline-sodic soils. DNA-fingerprints were obtained by PCR with BOX-A1R primer. The similarity of isolates was calculated using the UPGMA algorithm and Pearson's product moment correlation coefficient. The isolates were grouped in 36 clades (I to XXXVI), which were defined at 75% similarity.

Table 1
Genotypic and taxonomical features of broad range phosphate solubilizing bacteria (BRPSB).

Isolate	Sampling site	Box-PCR Cluster	n	Order	Potentially related genus/species
P163 P84 MA113	P - P -M	I	3	*Enterobacteriales*	*Pantoea eucalypti*
MA22	M	II	1	*Enterobacteriales*	unresolved
MA111 **MA28**	M	III	2	*Enterobacteriales*	unresolved
MB21 P75 **P76**	M - P - P	IV	3	*Enterobacteriales*	*Pantoea eucalypti*
MA58 MA59 MA53 MA56 **MA63**	M	V	5	*Enterobacteriales*	*Hafnia alvei*
MA66	M	VI	1	*Enterobacteriales*	*Pantoea eucalypti*
MA21	M	VII	1	*Enterobacteriales*	unresolved
MA35	M	VIII	1	*Enterobacteriales*	*Hafnia alvei*
MA67	M	IX	1	*Enterobacteriales*	*Enterobacter ludwigii*
MA41	M	X	1	*Enterobacteriales*	*Pantoea genus (except P. cypripedii)*
MA42	M	XI	1	*Enterobacteriales*	*Pantoea genus (except P. cypripedii)*
MA109	M	XII	1	*Enterobacteriales*	*Hafnia alvei*
P50	P	XIII	1	*Enterobacteriales*	*Erwinia genus*
MA104 MA99 **MA36** MA105 MA87 MA88	M	XIV	6	*Enterobacteriales*	*Hafnia alvei*
MA32	M	XV	1	*Enterobacteriales*	*Pantoea genus (except P. cypripedii)*
P102 **P137**	P	XVI	2	*Enterobacteriales*	*Enterobacter ludwigii*
P130	P	XVII	1	*Enterobacteriales*	*Enterobacter ludwigii*
P55, P99, **P101**, P85, P56, P86	P	XVIII	6	*Enterobacteriales*	*Enterobacter ludwigii*
P132, **P134**	P	XIX	2	*Enterobacteriales*	*Enterobacter ludwigii*
P128	P	XX	1	*Enterobacteriales*	*Enterobacter ludwigii*
MA65	M	XXI	1	*Enterobacteriales*	*Enterobacter ludwigii*
P138	P	XXII	1	*Enterobacteriales*	*Enterobacter ludwigii*
MB16, **MB18**	M	XXIII	2	*Enterobacteriales*	*Yokenella regensburgei*
P164	P	XXIV	1	*Enterobacteriales*	unresolved
P48, **P49**, P43, P44, P45	P	XXV	5	*Enterobacteriales*	*Kosakonia arachidis/K. oryzae/K. radicincitans*
P156, **P157**	P	XXVI	2	*Enterobacteriales*	unresolved
P177	P	XXVII	1	*Enterobacteriales*	unresolved
P188 P193 P187 P189 P191 P184 P186 P194	P	XXVIII	8	*Enterobacteriales*	unresolved
P133	P	XXIX	1	*Enterobacteriales*	unresolved
MB41 **MB48**	M	XXX	2	*Enterobacteriales*	unresolved
MA62	M	XXXI	1	*Enterobacteriales*	*Pantoea genus (except P. cypripedii)*
P61 P74 **P63**	P	XXXII	3	*Enterobacteriales*	*Pantoea eucalypti*
P173	P	XXXIII	1	*Enterobacteriales*	*Pantoea eucalypti*
P195	P	XXXIV	1	*Psedomonadales*	*Pseudomonas chlororaphis*
P198	P	XXXV	1	*Psedomonadales*	*Pseudomonas chlororaphis*
P215 P216	P	XXXVI	2	*Psedomonadales*	*Pseudomonas chlororaphis*

One isolate (in bold) from each cluster was selected and identified by 16S rRNA analysis. The accession numbers of the sequences are shown in Table S1. The sample sites are indicated as follows: P, Punta Indio and M, Manantiales. The letter n represents the number of isolates within each cluster.

groups belonging to the order *Enterobacteriales*, while the remaining isolates were related to the *Pseudomonadaceae* family, more specifically to members of the genus *Pseudomonas*. For each one of these families, a phylogenetic tree was constructed by comparing the 16S RNA sequences of the BRPSB with those of potentially related type strains (Fig. 2A and B; Supplementary Table S3). The analysis of 16S RNA sequences belonging to the order *Enterobacteria*les (Fig. 2A) revealed that most branches of the maximum likelihood tree have a low bootstrap support. When only branches supported by a cut-off value of 50% were considered (Supplementary Fig. S3), 5 of the isolates grouped with *Pantoea eucalypti*, while other 4 grouped –with weak bootstrap support– with members of the *Pantoea* genus (except for *P. cypripedii*). Eight isolates grouped with *Enterobacter ludwigii*, while other 3 isolates grouped with members of the genus *Enterobacter* (except *E. soli*) and *Leclercia adecarboxylata*. Similarly, weak bootstrap support was observed with the four isolates grouping with *Hafnia alvaei*, which belong to a cluster that tentatively may contain other 5 isolates as well as of members of several genera. MB48, showed no clear relationship with the other genus tested, while MB18 seemed to be related with *Yokenella regenseburgei*. P49 grouped with some members of the *Kosakonia* genus, while P50 grouped with members of the *Erwinia* genus. The information for individual isolates is summarized in Table 1. The diversity of genera and their grouping into different clusters (Fig. 2A, Supplementary Fig. S3) reflects the phylogenetic complexity that characterizes this family of Gram-negative bacteria. Regarding the strains belonging to the *Pseudomonadaceae* family, P195 (XXXIV), P198 (XXXV) and P215 (XXXVI) seemed to be related, with a low bootstrap support, to *Pseudomonas chlororaphis*, which belongs to the *Pseudomonas fluorescens* complex (Garrido-Sanz et al., 2017), (Fig. 2B, Table 1).

Several isolates from both sampling locations seemed to be related with either *P. eucalypti* and *E. ludwigii*, thus suggesting the capacity of members of these putative species to colonize *L tenuis* roots in alkaline-sodic soils. As previously mentioned, a former isolate of *P eucalypti* (strain M91) from soils with a lower pH, exhibited a good performance as a biofertilizer for *Lotus tenuis* in field assays carried out in a neutral soil. Hence, we decided to further investigate the PGPB properties of the *P. eucalypti* related strains isolated from restrictive alkaline-sodic environments and compare them with the promissory strain formerly identified. Given that for some bacterial groups the analysis of 16S rRNA is not sufficient for species identification, the housekeeping genes *rpo*B and *gyr*B were used as additional taxonomic markers, as suggested for members of the order *Enterobacteriales*, including the genus *Pantoea* (Adeolu et al., 2016; Brady et al., 2008, 2009), to test whether or not the strains P163, P76, MA66, P63 and P173, can be considered as members of the *P. eucalypti* species. Phylogenetic trees based on individual (Supplementary Figs. S1 and S2) and concatenated 16S, *rpo*B and *gyr*B fragments sequences (Fig. 3) were consistent with the phylogenies inferred for these isolates from the analysis based only on the 16S rRNA gene. This result together with the high sequence similarities found between the tested strains and *P. eucalypti* LMG 24197[T] (Table S7), support the phylogenetic assignment of these strains to the species *P. eucalypti*.

3.3. In vitro properties of the P. eucalypti strains

The solubilization ability displayed by the five *P. eucalypti* strains (P63, P163, P76, MA66 and P173) was estimated using a phosphorus solubilization index (PSI). For the reasons stated above the *P. eucalypti*

Fig. 2. Maximum-likelihood phylogenetic trees of the Broad Range Phosphate-Solubilizing Bacteria (BRPSB), based on type strains-16S rRNA gene sequences for *Enterobacteriales* (A) and *Pseudomonas* (B). The two trees were constructed on the base of 16S rRNA partial sequences (890 and 951 nucleotides respectively) using Kimura 2-parameter and gamma distribution models (G + I). Numbers indicate the results of the bootstrap analysis with 1000 replicates (bootstrap values below 50% are not shown). For visualization purposes, a condensed ML tree showing branches with 50% support is featured in Supplementary Fig. 3. GenBank accession numbers are indicated in parentheses (see supplemental Tables S5 and S6).
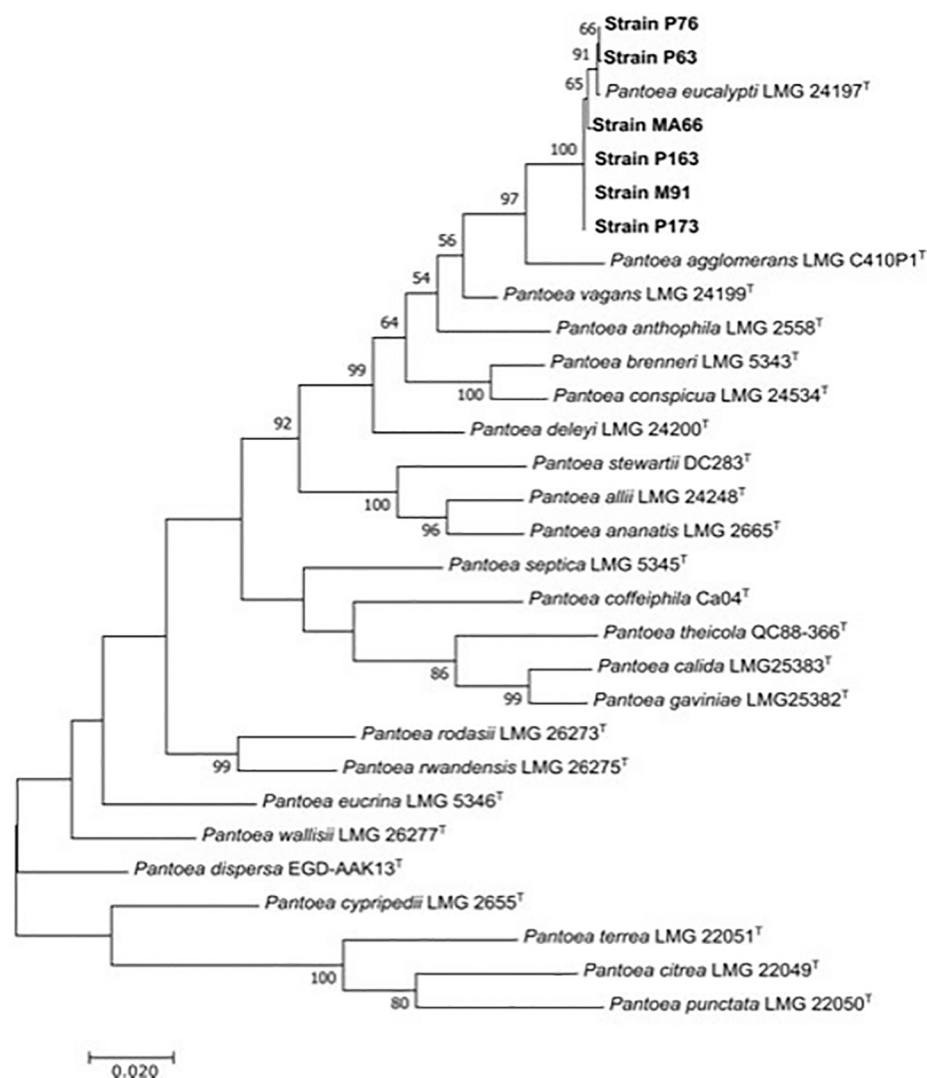
Fig. 3. Maximum-likelihood phylogenetic tree based on concatenated 16S rRNA, *gyr*B and *rpo*B (1271 nucleotides) gene sequences showing the position of P76, MA66, P63, P163, P173 and M91 strains within the genus *Pantoea*. The tree was constructed using Tamura 3-parameter and gamma distribution models (G + I). Bootstrap values calculated for 1000 replications are indicated at the nodes. Numbers indicate the results of the bootstrap analysis with 1000 replicates (bootstrap values below 50% are not shown). P76, MA66, P63, P163, P173 and M91 strains are indicated in bold. GenBank accession numbers of reference strains of genus *Pantoea* are indicated in parentheses (see Table S6).

M91 strain, previously isolated and characterized by our group, was included for comparative purposes. All the isolates have the capacity to solubilize phosphorus despite the negative effects exerted by alkaline-sodic conditions (Supplemental Table S8). The phosphate solubilization ability under neutral and alkaline-sodic conditions was further tested in liquid NBRIP medium at different incubation times. An interaction among the strain, the growth condition, and the time of exposure was detected, thus indicating a combined effect of these factors in determining the solubilization ability. We found that, at an early incubation time, soluble P tended to decrease –for most strains– under restrictive alkaline-sodic conditions. A time-dependent increase of soluble P in the culture medium was observed for all the conditions assayed. Although some differences among strains were observed at an early incubation stage (Fig. 4), they tended to disappear as long as the incubation time increased. It should be noted that, as a consequence of bacterial growth, the growth medium conditions could change during incubation. In this regard, it seems worth to note that the initial differences in the medium pH tended to disappear as incubation time progressed, similarly to the P solubilization pattern hereby described. Although these results suggest that caution should be exercised when drawing conclusions, they provide an additional confirmation that all the strains have the capability to solubilize P at neutral and alkaline-sodic conditions and indicate that this capability can be sustained during long incubation periods (Fig. 4).

We also investigated if the *P. eucalypti* strains analyzed in the present work exhibit other traits potentially related with plant growth promotion. All the strains produced siderophores under both control and alkaline-sodic conditions, with areas ranging between 0.92 and 3.09 cm^2 and 1.40 to 3.47 cm^2 under control and alkaline-sodic conditions, respectively (Supplemental Table S8). In turn, all the strains produced detectable levels of IAA, with P163, MA66, P76, P63 and P173 producing much higher levels (34.05 to 37.21 µg/mL) than M91 (5.65 µg/mL) (Supplemental Table S8). Taken together, our results indicate that the group of *P. eucalypti* strains identified in this work share some *in vitro* features, namely the capacity to solubilize P and to produce IAA and siderophores, which could impact on plant growth as well as on plant mineral nutrient dynamics.

3.4. Effect of different P eucalypti strains on Lotus tenuis growth, nutrition and symbiotic rhizobial interaction in an alkaline-sodic soil

Sowing *L. tenuis* seeds in the field involves inoculation with nitrogen-fixing symbiotic rhizobia. Therefore, each *P. eucalypti* strain used in this assay was tested in combination with a native rhizobial strain (*Mesorhizobium sanjuanii* BSA136). Plants inoculated only with the rhizobial strain (R), were used as control. It should be mentioned that, prior to this
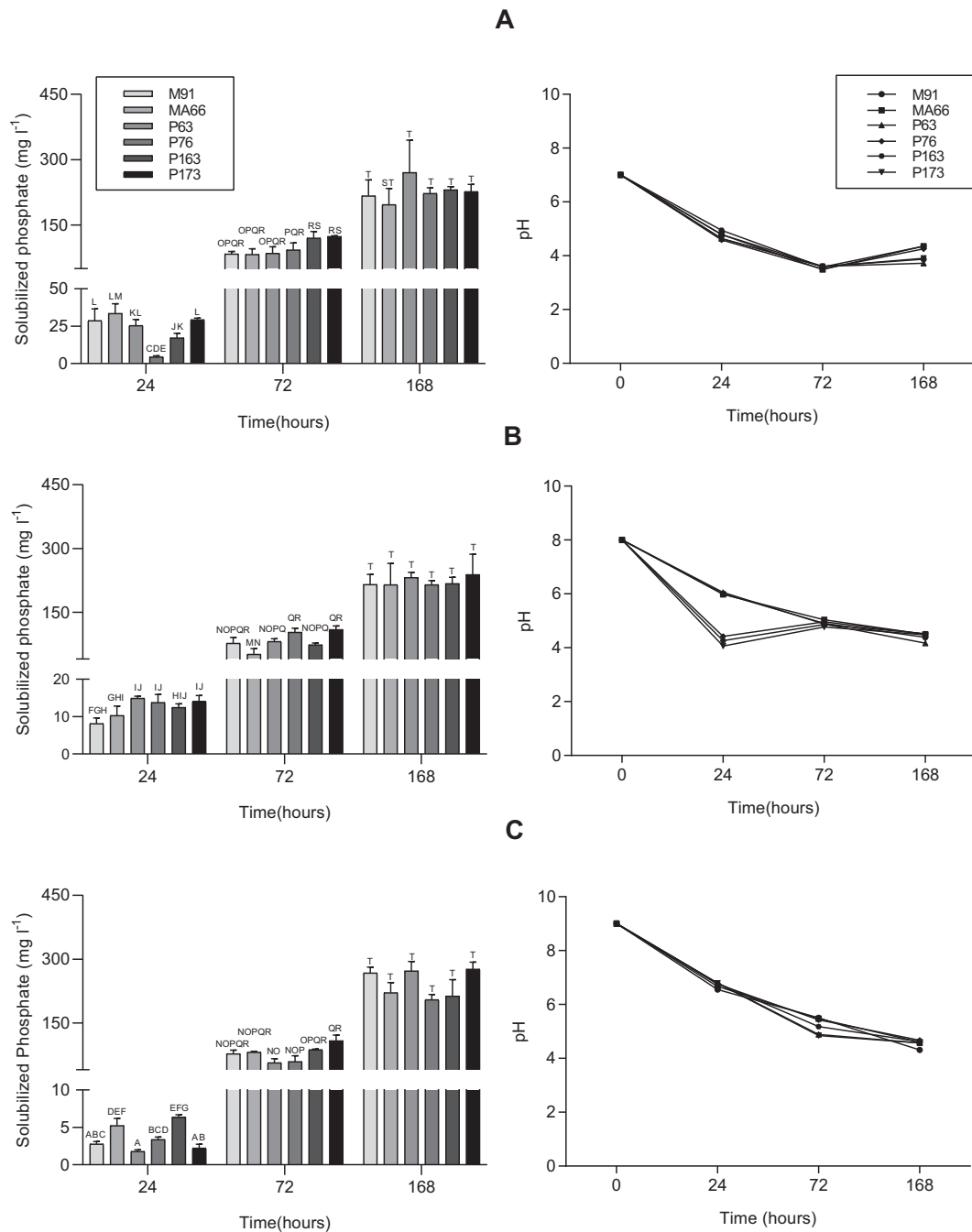
Fig. 4. Phosphate solubilization ability of *Pantoea eucalypti* strains in liquid medium under neutral and alkaline-sodic conditions.
The P-solubilization ability (left panel) and the pH in the media (right panel) were evaluated in liquid NBRIP medium with tricalcium phosphate as insoluble phosphate source at different incubation times in three conditions. A: pH 7.0, B: pH 8.0 + 200 mM Na$^+$ and C: pH 9.0 + 200 mM Na$^+$. The bars represent the means of 3 replicates ±SE. P-solubilization data were analyzed by factorial ANOVA followed by Tukey's multiple-comparison test. Different letters indicate statistically significant differences ($P \leq 0.05$).

experiment, the compatibility between *Mesorhizobium sanjuanii* BSA136 and *P. eucalypti* strains was analyzed in order to test the existence of antagonistic effects between them. After 5 days of incubation, no growth inhibition was exerted by any of the tested strains, indicating that both organisms display *in vitro* compatibility.

A significant effect of the strains (*i.e.* treatments) on dry matter accumulation was detected. Post-hoc analysis indicated that the dry matter accumulated by co-inoculated plants showed no significant differences relative to the control treatment (R). A similar pattern was observed for shoot dry weight (DWsh) (Table 2). Interestingly, the presence of the strain P76 in the co-inoculation treatment reduced total and shoot DW compared to that of plants co-inoculated with the R + M91 or R + P163 mixtures. These data indicate that different strains of *P. eucalypti* exert differential effects on *L. tenuis* growth. In addition, a significant effect of *P. eucalypti* strains on nodule biomass accumulation by *M. sanjuanii* was also detected. Nodule dry weight (DWn) values for plants treated with the co-inoculation mixtures R + MA66, R + P173, R + P63 and R + P163 were similar to those determined in R plants. Interestingly, the combination of R and M91 increased the DWn (62.9%) when compared to the R treatment (Table 2). On the contrary, DWn was negatively affected by the presence of the P76 strain in the co-inoculation treatment, causing a strong reduction (77.8%) compared to control plants. These results indicate that different strains of *P. eucalypti* differentially affect the dry matter associated to the nodules of *M. sanjuanii* in *L. tenuis* plants and, therefore, opened the possibility that some of them may interfere with the ability to incorporate nitrogen into the plant through this mutualistic symbiotic relationship. Based on the above results and to test this hypothesis, a subsequent analysis was performed to study the concentration and accumulation of nitrogen in plants inoculated with rhizobia (R) and three of the five previously mentioned treatments, which were selected on the basis of the contrasting phenotypes observed in plants and nodules. Therefore, we analyzed plants exposed to R, R + M91, R + P76 and R + P163 treatments.

The concentration of nitrogen in both shoots and roots of co-inoculated plants was significantly affected by the treatment. In all co-inoculation treatments it was similar to that of plants inoculated only with the rhizobial strain regardless of the *P. eucalypti* strain (Supplemental Fig. S4). Strikingly, a lower value for the concentration of this major element (36.4 and 20.78% for shoots and roots, respectively) was observed in plants co-inoculated with R + P76 compared with plants treated with the R + M91 mixture. In turn, the total amount of nitrogen accumulated per plant was also significantly affected, being similar for R + P163 and R plants, while for R + M91 a 61.64% increase was detected relative to R plants, indicating that the M91 strain enhances the acquisition of nitrogen (Fig. 5). Conversely, the inoculation of the R + P76 mixture had the opposite effect on the total accumulation of nitrogen per plant, displaying a 34% reduction relative to R plants and even lower relative to plants co-inoculated with R + M91 or R + P163 (59.15% and 45.7% respectively) (Fig. 5). When nitrogen accumulation in the aerial part was compared among the different inoculation treatments, a significant increase (69.39%) was observed for plants treated

with the co-inoculation mixture R + M91, compared to R plants (data not shown, see the shoot dry weight data posted in Table 2 and concentration data for nitrogen in Supplemental Fig. S4). The effect of co-inoculation treatments on the concentration and amount of P in the plant was also analyzed (Supplemental Fig. S4, Fig. 5). No effect of the treatment was detected for the concentration of phosphorus in the shoot, while for roots a significant effect was observed. For this organ, P concentration was not affected by the co-inoculation treatments R + M91 and R + P163, as compared to R plants. Interestingly, the roots of plants co-inoculated with R + P76 mixture displayed a high P concentration relative to R and R + P163 plants (Supplemental Fig. S4). On the other hand, the treatment exerted a significant effect on total phosphorus accumulation per plant. Plants treated with R + M91 mixture displayed an increase of 47.89% relative to R plants (Fig. 5). In turn, R + P76 treated plants displayed a lower accumulation of P than R + M91.

In order to obtain a more complete picture regarding the effect of different co-inoculation mixtures on the elemental composition and acquisition of mineral nutrients in *L. tenuis*, the accumulation of other elements, including K, Ca, Mg, Na, Fe, Zn, Cu, and Mn, was examined (Fig. 5). It was found that the accumulation of most of the elements (8 of 10), including N and P, was significantly higher for plants co-inoculated with the combination of R and M91 than in those co-inoculated with R + P76, which can be linked –at least partially– to the above mentioned differences in dry weight accumulation. In turn, plants treated with the mix R + M91 showed a tendency to accumulate more N, P and Zn compared with R plants. In addition our results also indicated that the R + P163 co-inoculation treatment exerted a positive effect on Zn accumulation, relative to R plants (Fig. 5).

The next question to be addressed was whether or not the differences in the amount of each nutrient accumulated actually lead to a different nutritional status in both plant organs. When the concentration of all the elements studied was examined for shoots and roots, significant effects of the treatment were observed for Zn and Mn in shoots and for Zn and Mg in roots, in addition to those already mentioned for P or N. The concentration of Mg tended to be lower in roots of plants co-inoculated as compared with R plants. On the contrary, the concentration of Zn in this organ was significantly enhanced by all the co-inoculation treatments, suggesting a major effect of the *P. eucalypti* strains on the internal status of this micronutrient.

4. Discussion

Over the last decades, increasing evidence supports a key role for microorganisms in determining plant phenotypic plasticity to a changing environment while bacterial- prospecting studies conducted over a wide range of locations revealed the plant rhizosphere as a major source of microbial resources (Bakker et al., 2018; Goh et al., 2013). A key observation emerging from those studies is that composition of root-associated microbiota may be influenced by the environment as well as for the plant species (Compant et al., 2019), although no all ecosystems have been equally examined. In this regard, alkaline-sodic soils are among the least explored environments, with only a few studies

Table 2
Effect PSB inoculation on dry matter accumulation in *Lotus tenuis* plants interacting with nitrogen-fixing rhizobia.

Variable	Treatment						
	R + P163	R + P76	R + MA66	R + P63	R + P173	R + M91	R
DW$_{sh}$	0.73 ± 0.05^{bc}	0.48 ± 0.05^{a}	0.52 ± 0.05^{ab}	0.53 ± 0.05^{ab}	0.68 ± 0.05^{abc}	0.76 ± 0.03^{c}	0.56 ± 0.05^{abc}
DW$_r$	0.32 ± 0.04	0.26 ± 0.03	0.28 ± 0.02	0.28 ± 0.04	0.35 ± 0.03	0.34 ± 0.02	0.28 ± 0.03
DW$_n$	0.035 ± 0.003^{bc}	0.006 ± 0.003^{a}	0.026 ± 0.003^{b}	0.028 ± 0.003^{b}	0.032 ± 0.003^{bc}	0.044 ± 0.003^{c}	0.027 ± 0.003^{b}
DW$_t$	1.08 ± 0.07^{bc}	0.74 ± 0.07^{a}	0.81 ± 0.07^{ab}	0.83 ± 0.07^{abc}	1.06 ± 0.07^{abc}	1.14 ± 0.07^{c}	0.87 ± 0.07^{abc}

DW$_{sh}$: Shoot dry weight (g), **DW$_r$:** Root dry weight (g), **DW$_n$:** Nodule dry weight (g), **DW$_t$:** Total dry weight (g). Results are means of 6–8 biological replicates ±SE. The values were analyzed by one-way ANOVA, followed by Tukey's multiple-comparison test. Different letters indicate statistically significant differences (p ≤ 0.05) in growing values within rows.
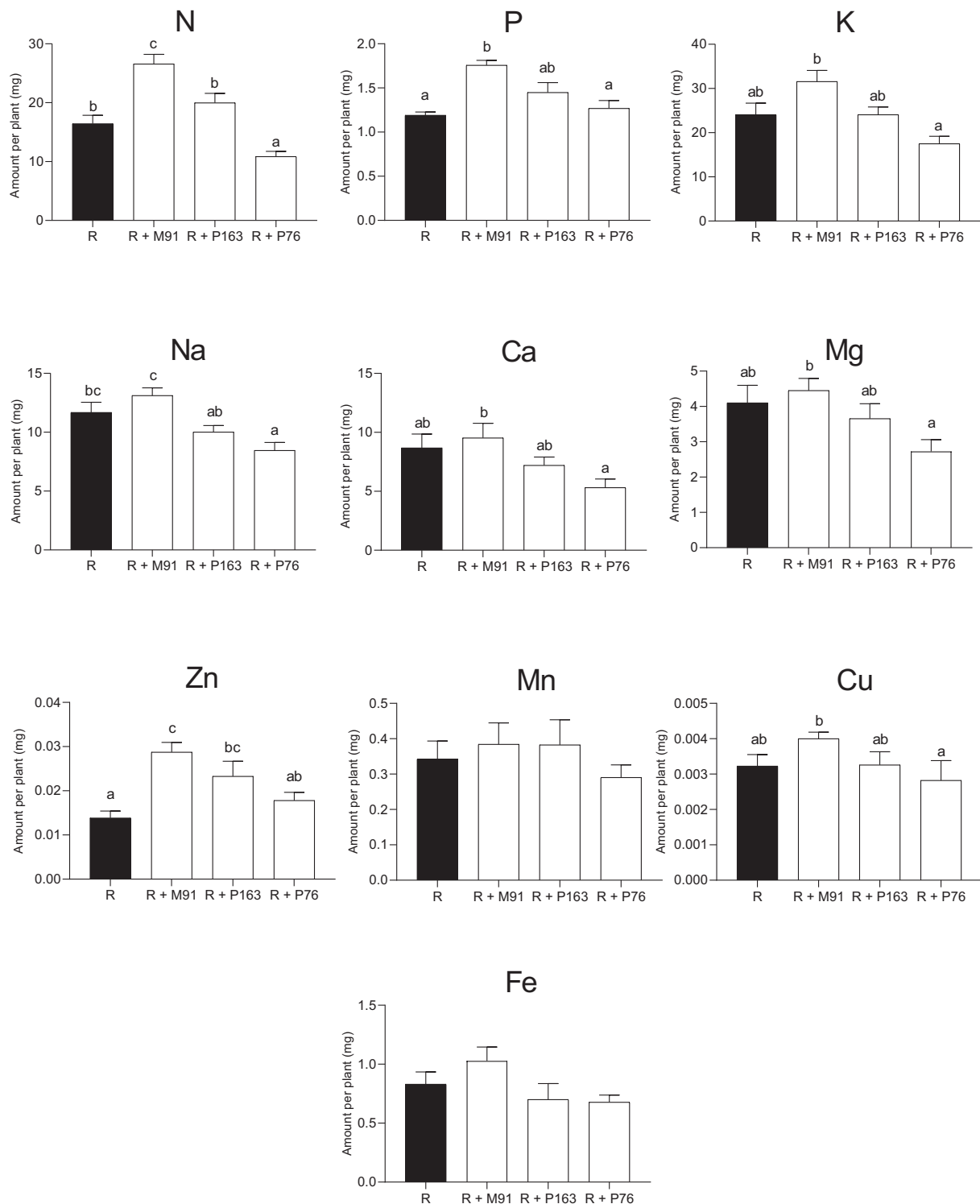
Fig. 5. Total amount of mineral nutrient in *Lotus tenuis* plants inoculated with *P. eucalypti* - *M. sanjuanii* BSA136 consortia. The total amount of each one of the elements measured in whole plants is shown. Results correspond to the mean of 6–8 biological replicates ±SE. The values were analyzed by one way ANOVA, followed by Tukey's multiple-comparison test. Different letters indicate statistically significant differences ($p \leq 0.05$) between treatments.

surveying their rhizosphere biodiversity. Noticeably, in these soils, the availability of P and some micronutrients is a major limiting factor for agricultural productivity. However, knowledge about the cultivable microbiota with the ability to solubilize P in alkaline-sodic environments, even growing, remains scarce (Anam et al., 2019; Banerjee et al., 2010; Dixit et al., 2020; Nautiyal et al., 2000). Recent studies unveiled that the structure of P-solubilizing bacterial communities is strongly

affected by soil pH gradients (Zheng et al., 2019). As aforementioned, there is a wide range of pH variation in soils of the flooding pampas (Perelman et al., 2007; Stoffella et al., 1998). Thus, phosphate-solubilization over a wide range of pH is a desirable trait for PSB to be used in the formulations of biofertilizers for this region as well as for other locations. However, the taxonomic diversity of microorganisms native from alkaline-sodic soils that display this capacity has not been

previously assessed. In this work we focused on prospecting for cultivable PSB that colonize the rhizosphere of naturalized *Lotus tenuis* plants in alkaline-sodic soils of the flooding pampa. The screening hereby presented provide the first overview about the diversity of these rhizospheric PSB and unveiled that a significant fraction (74 out of 299) of isolates (designated as BRPSB) are able to solubilize tricalcium phosphate at neutral and a broad range of alkaline-sodic conditions. However, our results indicate that this is not a universal trait among bacteria from the alkaline-sodic environments examined, since an even larger fraction (225 out of 299 isolates) only formed solubilization haloes within a narrow range of pH. Noticeably, the subset of 74 isolates of BRPSB displayed a limited degree of taxonomic variation. Despite numerous reports suggesting that the ability to solubilize phosphate is not limited to a few bacterial groups but rather extends to taxa belonging to various phyla (Zheng et al., 2017), the 16S rRNA phylogenetic analysis of this functional subset of isolates showed that it was composed exclusively by members of the *Proteobacteria* phylum. In this regard, it should be noted that numerous evidences indicate that *Proteobacteria* is a major phylum in rhizospheric environments (García-Salamanca et al., 2013; Zgadzaj et al., 2016). Thus, the possibility that the narrow range of high taxonomic categories here found for BRPSB could be –at least partially– a consequence of the usually high abundance of Proteobacteria in the rhizosphere should be not ruled out. However, it should also be considered that our findings might reflect a pattern specific to alkaline-sodic soils of the flooding pampa here tested, since members of other phyla isolated from alkaline environments of West Bengal, were able to solubilize P over a wide pH range (Banerjee et al., 2010). Alternatively, it could be a consequence of the influence of the plant species, which is considered a key factor contributing to the recruitment and establishment of microbial communities in the rhizosphere (Compant et al., 2019).

Some strains of symbiotic rhizobia belonging to the Alphaproteobacteria group have been shown to solubilize P (Peix et al., 2001; Rosas et al., 2006). It is worth to point out that, despite the well-known high abundance of rhizobia in the rhizosphere of legumes (Hartman et al., 2017), no rhizobial genus was detected within the BRPSB group in the present study. The genus *Mesorhizobium* comprises species of *L. tenuis* and chickpea-nodulating rhizobia, among others. While some chickpea rhizobia were shown to solubilize P (Peix et al., 2001), a low P solubilization activity was found in a large collection of *L. tenuis* mesorhizobia (Estrella et al., 2009, our unpublished results). These findings highlight the importance of investigating PSB-*Mesorhizobium*-*L. tenuis* interactions to take advantage of the potential benefits of both types of microorganisms in the nutrition and growth of this legume.

Among the BRSPB strains found in this study, we focused on members of the *Pantoea* genus as previous studies made by us (Campestre et al., 2016; Castagno et al., 2011) as well as by other authors (Son et al., 2006) indicated their capability to dwell in adverse environments in association with legumes. A further characterization of a subset of the here identified BRSPB belonging to *P. eucalypti* species and the previously found *P. eucalypti* M91 strain, revealed that, in addition to the ability to solubilize P under neutral and alkaline-sodic conditions, they produce siderophores and indole acetic acid and showed compatibility with *Mesorhizobium sanjuanii* BSA136 under *in vitro* culture conditions. In legumes, combined inoculation with rhizobia and phosphate-solubilizing bacteria may exert a positive impact on nodule number and weight, plant dry weight accumulation, as well as on the total nitrogen incorporated by plants (Guiñazú et al., 2010; Rosas et al., 2006). Noticeably, in most studies, the effects of rhizobia-PSB co-inoculation have been investigated by comparing PSB strains belonging to different genera/species with no particular focus on intra-specific comparisons. The set of closely related BRPSB identified in this work, provided an opportunity to analyze the impact of strains sharing genetic, and *in vitro* common functional traits on the growth and nutrient acquisition of a nodulating legume growing in alkaline-sodic soils. Despite the above-mentioned similarities exhibited by these strains *in vitro*, different

effects on plant growth, nodulation and nutrient accumulation were detected. This finding suggests that closely related bacteria sharing key *in vitro* growth-promoting properties do not necessarily exert similar effects on symbiotic nodulation and plant performance. Thus, the beneficial effects cannot be predicted exclusively on the basis of their *in vitro* PGPR attributes, and complementary tests on plants are necessary to select adequate strains for field inoculation of *L. tenuis* plants in combination with nitrogen-fixing rhizobia.

A striking result from the co-inoculation assay was that the allocation of dry matter to the nodules sharply differed among the plants treated with different *P. eucalypti* strains; thus indicating that even when they all show *in vitro* compatibility with *M. sanjuanii* BSA136, *in planta* symbiosis was differentially affected. A major issue to be addressed in future studies is to disclose the mechanisms implicated in this differential host investment in nodules. Since the plant's hormonal balance is pivotal for nodule development (Liu et al., 2018) and it may be affected by multiple signals, including the production of hormones and other signaling molecules (Simontacchi et al., 2013), the possibility that more than a single pathway is affected by different *P. eucalypti* strains in tripartite interactions, should be considered. In this regard, hormone production may not be the same for all strains, as indirectly suggested –although not tested *in planta*– by the different *in vitro* IAA production found for M91 relative to the rest of the strains. Other possibilities should be also taken into consideration as different processes could drive the positive and negative effects of PSB. In this regard, studies on the tripartite interaction between the pathogenic bacteria *Pseudomonas syringae* pv *tomato* DC3000, *Sinorhizobium meliloti* and the host *Medicago truncatula*, revealed an antagonistic role of plant innate immunity in symbiosis (Chen et al., 2017). The reduction of biomass allocated to the nodules found in plants co-inoculated with *M. sanjuanii* BSA136 and P76 are indicative of a negative interaction. Whether the effect exerted by P76 involves the modulation of plant innate immunity is worth to be explored.

A major issue in modern agriculture is to improve the efficiency in the acquisition and utilization of mineral nutrients, which may help to reduce economic costs as well as to minimize negative impacts on ecosystems (Nieves-Cordones et al., 2020). Biofertilizers are considered a valuable tool to improve the above-mentioned efficiencies. Results here shown, disclose novel and contrasting effects of *P. eucalypti* strains on nutrient acquisition. In parallel to this work, Campestre et al. (2020) provided an assessment on the influence of the *P. eucalypti* M91 strain alone in a full nutrient hydroponic medium, showing, under alkaline conditions, no effect of this strain on *L. tenuis* growth nor on the concentration of most nutrients analyzed, while the effect of this strain on nutrient acquisition was not quantified. Here, we showed that inoculation of *L. tenuis* plants with a mixture of *M. sanjuanii* BSA136 and *P. eucalypti* M91 grown in a low P-availability alkaline soil, did not improve plant growth as compared to inoculation with single rhizobia. However, plants co-inoculated with *M. sanjuanii* BSA136 and *P. eucalypti* M91 displayed a superior capability to accumulate N, P and Zn. Therefore, the use of this strain in co-inoculation with *M. sanjuanii* BSA136 could help to simultaneously enhance the efficiency for the acquisition of these three nutrients in alkaline-sodic soils. In turn, P163 could be an adequate alternative partner for co-inoculation with *M. sanjuanii* BSA136 to improve Zn acquisition in *L. tenuis*. Importantly, our work disclose a major difference in the total accumulation of several nutrients by plants co-inoculated with different *P. eucalypti* strains, particularly in comparisons between M91 and P76, thus indicating that even highly related *P. eucalypti* strains may exert different nutritional impact in plants. The dynamics of nutrient accumulation by plants is a complex process (Epstein and Bloom, 2005) that, among other factors, depends on the availability of the nutrient in the root absorption zone, the specific capacity for uptake per unit of root weight, the size and architecture of the root system, dilution and concentration effects derived from growth differences, as well as on the allocation pattern within the plant. Although all the strains under evaluation displayed *in vitro* attributes

that would potentially affect some of the aforesaid *in planta* processes, their precise effect need to be further examined in order to disclose the precise mechanisms by which nutrient acquisition in *L. tenuis* is differentially affected by each *P. eucalypti* strain.

An important aspect to be considered in biofertilizers design for pastures is not only their capacity to ensure an adequate provision of major nutrients, but also whether or not they exert an impact on the forage quality (Mishra et al., 2008). In fact, co- inoculation of *P. eucalypti* strains M91 and P163 with *Mesorhizobium sanjuanii* BSA136 showed no negative effects on plant grown in alkaline soil. However, a reduced concentration of Mg in roots of *L. tenuis* plants treated with *P. eucalypti* strains was found. The results indicate that *P. eucalypti* has the capability to modulate the internal Mg status of roots, without affecting its acquisition. In this context it seems relevant to note that our results also show that inoculation with any of the *P. eucalypti* strains here assayed does not lead to a reduction of Mg concentration in shoots, which is critical in zones devoted to cattle raising where hypomagnesemia is known to be a major problem (Doncel et al., 2019).

The contrasting different responses observed in *L. tenuis* plants when inoculated with the *P. eucalypti* M91 and P76 strains highlight the complexity and specificity of plant-microbe interactions and reveal the need to perform in deep studies to better understand the different observed phenotypes and the possible mechanisms involved. Importantly, the differential effects exerted by highly related strains of *P. eucalypti* on *L. tenuis* plants unveil the importance of evaluating the efficacy of tripartite interactions between PSB, rhizobia and legumes as a fundamental strategy for the selection of microorganisms to be used as biofertilizers. In addition, large screenings in different alkaline-sodic soils of the world, as well as of other plant species in the environments here studied, may help to understand the basis for the restricted taxonomic diversity of BRPSB native of these environments.

5. Conclusions

The present study responded, according to our starting hypothesis, that the rhizospheric microbial community of *L. tenuis* plants naturally grown in alkaline sodic soils of the flooding pampa harbors cultivable bacterial species capable of solubilizing P under a wide range of alkaline-sodic conditions. This group of bacteria contained a high proportion of species of the order *Enterobacteriales*, as well as a minor proportion of strains of *Pseudomonas chlororaphis* (family *Pseudomonadaceae*).

A comparative analysis of isolates belonging to the *P. eucalypti* species allowed us to test our second hypothesis and revealed that, despite sharing key *in vitro* properties, they exert highly variable outcomes on *Lotus tenuis* performance when applied in combination with nitrogen-fixing rhizobia. Our results indicate the complexity of plant-microorganism interactions and show that evaluation of the tripartite interaction PSB-rhizobia-legume is an unavoidable step as part of a process of selection of strains to be used as biofertilizers.

Supplementary data to this article can be found online at https://doi. org/10.1016/j.apsoil.2021.104125.

Declaration of competing interest

The authors have no conflict of interest to declare.

References

Adcock, D., McNeill, A.M., McDonald, G.K., Armstrong, R.D., 2007. Subsoil constraints to crop production on neutral and alkaline soils in south-eastern Australia: a review of current knowledge and management strategies. Aust. J. Exp. Agric. 47, 1245–1261.

Adeolu, M., Alnajar, S., Naushad, S., Gupta, R.S., 2016. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. Nov., Morgane. Int. J. Syst. Evol. Microbiol. 66, 5575–5599. https://doi.org/10.1099/ijsem.0.001485.

Alori, E.T., Glick, B.R., Babalola, O.O., 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front. Microbiol. 8, 1–8. https://doi.org/10.3389/fmicb.2017.00971.

Anam, G.B., Reddy, M.S., Ahn, Y.H., 2019. Characterization of Trichoderma asperellum RM-28 for its sodic/saline-alkali tolerance and plant growth promoting activities to alleviate toxicity of red mud. Sci. Total Environ. 662, 462–469. https://doi.org/10.1016/j.scitotenv.2019.01.279.

Bakker, P.A.H.M., Pieterse, C.M.J., de Jonge, R., Berendsen, R.L., 2018. The soil-borne legacy. Cell 172, 1178–1180. https://doi.org/10.1016/j.cell.2018.02.024.

Banerjee, S., Palit, R., Sengupta, C., Standing, D., 2010. Stress induced phosphate solubilization by arthrobacter sp. and bacillus sp. isolated from tomato rhizosphere. Aust. J. Crop. Sci. 4, 378–383.

Bradstreet, R.B., 1954. Kjeldahl method for organic nitrogen. Anal. Chem. 26 (1), 185–187. https://doi.org/10.1021/ac60085a028.

Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., Coutinho, T., 2008. Phylogeny and identification of Pantoea species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Syst. Appl. Microbiol. 31, 447–460. https://doi.org/10.1016/j.syapm.2008.09.004.

Brady, C.L., Venter, S.N., Cleenwerck, I., Engelbeen, K., Vancanneyt, M., Swings, J., Coutinho, T.A., 2009. Pantoea vagans sp. nov., Pantoea eucalypti sp. nov., Pantoea deleyi sp. nov. and Pantoea anthophila sp. nov. Int. J. Syst. Evol. Microbiol. 59, 2339–2345. https://doi.org/10.1099/ijs.0.009241-0.

Bray, R.H., Kurtz, L.T., 1945. Determination of Total organic and available forms of phosphorus in soils. Soil Sci. 59, 39–45.

Campestre, M.P., Castagno, L.N., Estrella, M.J., Ruiz, O.A., 2016. Lotus japonicus plants of the Gifu B-129 ecotype subjected to alkaline stress improve their Fe^{2+} bio-availability through inoculation with Pantoea eucalypti M91. J. Plant Physiol. 192. https://doi.org/10.1016/j.jplph.2016.01.001.

Campestre, M.P., Castagno, L.N., Antonelli, C.J., Maguire, V.G., Escaray, F.J., Ruiz, O.A., 2020. Characterization of alkaline stress tolerance mechanisms in Lotus forage species modulated by Pantoea eucalypti. bioRxiv. https://doi.org/10.1101/2020.06.06.138230, 2020.06.06.138230.

Castagno, L.N., Estrella, M.J., Sannazzaro, A.I., Grassano, A.E., Ruiz, O.A., 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. https://doi.org/10.1111/j.1365-2672.2011.04968.x.

Castagno, L.N., García, I.V., Sannazzaro, A.I., Bailleres, M., Ruiz, O.A., Mendoza, R.E., Estrella, M.J., 2014. 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. Plant Soil 385, 357–371. https://doi.org/10.1007/s11104-014-2237-z.

Chen, T., Duan, L., Zhou, B., Yu, H., Zhu, H., Cao, Y., Zhang, Z., 2017. Interplay of pathogen-induced defense responses and symbiotic establishment in Medicago truncatula. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.00973.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42, D633–D642. https://doi.org/10.1093/nar/gkt1244.

Compant, S., Samad, A., Faist, H., Sessitsch, A., 2019. A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. J. Adv. Res. 19, 29–37. https://doi.org/10.1016/j.jare.2019.03.004.

Dauga, C., 2002. Evolution of the gyrB gene the molecular phylogeny of Enterobacteriaceae: a model molecule for molecular systematic studies. Int. J. Syst. Evol. Microbiol. 52, 531–547.

Di Rienzo, J., Balzarini, M., Robledo, C., Casanoves, F., Gonzales, L., Tablada, E., 2008. InfoStat Software Manual del Usuario. FCA Univ. Nac. Córdoba, p. 334.

Dixit, V.K., Misra, S., Mishra, S.K., Tewari, S.K., Joshi, N., Chauhan, P.S., 2020. Characterization of plant growth-promoting alkalotolerant Alcaligenes and Bacillus strains for mitigating the alkaline stress in Zea mays. Antonie van Leeuwenhoek. Int.

J. Gen. Mol. Microbiol 113, 889–905. https://doi.org/10.1007/s10482-020-01399-1.

Doncel, B., Capelesso, A., Giannitti, F., Cajarville, C., Macías-Rioseco, M., Silveira, C., Costa, R.A., Riet-Correa, F., 2019. Hypomagnesemia in dairy cattle in Uruguay. Pesqui. Vet. Bras. 39, 564–572. https://doi.org/10.1590/1678-5150-PVB-6215.

Epstein, E., Bloom, A.J., 2005. Mineral Nutrition of Plants : Principles and Perspectives. Sinauer Associates, Inc., Sunderland, Mass.

Estrella, M.J., Muñoz, S., Soto, M.J., Ruiz, O., Sanjuán, J., 2009. Genetic diversity and host range of rhizobia nodulating *Lotus tenuis* in typical soils of the Salado River Basin (Argentina). Appl. Environ. Microbiol. 75 https://doi.org/10.1128/AEM.02405-08.

Etesami, H., Beattie, G.A., 2018. Mining halophytes for plant growth-promoting halotolerant bacteria to enhance the salinity tolerance of non-halophytic crops. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.00148.

Eynard, A., Lal, R., Wiebe, K., 2005. Crop response in salt-affected soils. J. Sustain. Agric. 27 (1), 5–50. https://doi.org/10.1300/J064v27n01_03.

García-Salamanca, A., Molina-Henares, M.A., van Dillewijn, P., Solano, J., Pizarro-Tobías, P., Roca, A., Duque, E., Ramos, J.L., 2013. Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. Microb. Biotechnol. 6, 36–44. https://doi.org/10.1111/j.1751-7915.2012.00358.x.

Garrido-Sanz, D., Arrebola, E., Martínez-Granero, F., García-Méndez, S., Muriel, C., Blanco-Romero, E., Martín, M., Rivilla, R., Redondo-Nieto, M., 2017. Classification of isolates from the *Pseudomonas fluorescens* complex into phylogenomic groups based on group-specific markers. Front. Microbiol. 8, 1–10. https://doi.org/10.3389/fmicb.2017.00413.

Glick, B.R., 2012. Plant Growth-Promoting Bacteria : Mechanisms and Applications, p. 2012.

Goh, C.H., Veliz Vallejos, D.F., Nicotra, A.B., Mathesius, U., 2013. The impact of beneficial plant-associated microbes on plant phenotypic plasticity. J. Chem. Ecol. 39, 826–839. https://doi.org/10.1007/s10886-013-0326-8.

Guiñazú, L.B., Andrés, J.A., Del Papa, M.F., Pistorio, M., Rosas, S.B., 2010. Response of alfalfa (Medicago sativa L.) to single and mixed inoculation with phosphate-solubilizing bacteria and *Sinorhizobium meliloti*. Biol. Fertil. Soils 46, 185–190. https://doi.org/10.1007/s00374-009-0408-5.

Hartman, K., van der Heijden, M.G.A., Roussely-Provent, V., Walser, J.C., Schlaeppi, K., 2017. Deciphering composition and function of the root microbiome of a legume plant. Microbiome 5, 1–13. https://doi.org/10.1186/s40168-016-0220-z.

He, A., Niu, S.Q., Zhao, Q., Li, Y.S., Gou, J.Y., Gao, H.J., Suo, S.Z., Zhang, J.L., 2018. Induced salt tolerance of perennial ryegrass by a novel bacterium strain from the rhizosphere of a desert shrub *Haloxylon ammodendron*. Int. J. Mol. Sci. 19, 1–19. https://doi.org/10.3390/ijms19020469.

Hopkins, B.G., Ellsworth, J., 2005. Phosphorus availability with alkaline/calcareous soil. West. Nutr. Manag. Conf 88–93.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Liu, H., Zhang, C., Yang, J., Yu, N., Wang, E., 2018. Hormone modulation of legume-rhizobial symbiosis. J. Integr. Plant Biol. 60, 632–648. https://doi.org/10.1111/jipb.12653.

Liu, J., Tang, L., Gao, H., Zhang, M., Guo, C., 2019. Enhancement of alfalfa yield and quality by plant growth-promoting rhizobacteria under saline-alkali conditions. J. Sci. Food Agric. 99, 281–289. https://doi.org/10.1002/jsfa.9185.

Lorite, M.J., Estrella, M.J., Escaray, F.J., Sannazzaro, A., Videira E Castro, I.M., Monza, J., Sanjuán, J., León-Barrios, M., 2018. The rhizobia-*Lotus* symbioses: deeply specific and widely diverse. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.02055.

Mishra, S., Sharma, S., Vasudevan, P., 2008. Comparative effect of biofertilizers on fodder production and quality in guinea grass (*Panicum maximum* Jacq.). J. Sci. Food Agric. 88, 1667–1673.

Nautiyal, C.S., 1999. An eficiente microbiological growth médium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265–270. https://doi.org/10.1111/j.1574-6968.1999.tb13383.x.

Nautiyal, C.S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., Verma, D., 2000. Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiol. Lett. 182, 291–296. https://doi.org/10.1016/S0378-1097(99)00605-9.

Nieves-Cordones, M., Rubio, F., Santa-María, G.E., 2020. Editorial: nutrient use-efficiency in plants: an integrative approach. Front. Plant Sci. 11, 623976 https://doi.org/10.3389/fpls.2020.623976.

Owen, D., Williams, A.P., Griffith, G.W., Withers, P.J.A., 2015. Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Appl. Soil Ecol. 86, 41–54. https://doi.org/10.1016/j.apsoil.2014.09.012.

Peix, A., Rivas-Boyero, A.A., Mateos, P.F., Rodriguez-Barrueco, C., Martínez-Molina, E., Velazquez, E., 2001. Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. Soil Biol. Biochem. 33, 103–110. https://doi.org/10.1016/S0038-0717(00)00120-6.

Perelman, S.B., Chaneton, E.J., Batista, W.B., Burkart, S.E., León, R.J.C., 2007. Habitat stress, species pool size and biotic resistance influence exotic plant richness in the flooding Pampa grasslands. J. Ecol. 95, 662–673. https://doi.org/10.1111/j.1365-2745.2007.01255.x.

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L.S., Fernández, F.J., 2007. O-CAS, a fast and universal method for siderophore detection. J. Microbiol. Methods 70, 127–131. https://doi.org/10.1016/j.mimet.2007.03.023.

Pla-Sentís, I., 2014. Advances in the prognosis of soil sodicity under dryland irrigated conditions. Int. Soil Water Conserv. Res. 2, 50–63. https://doi.org/10.1016/S2095-6339(15)30058-7.

Rosas, S.B., Andrés, J.A., Rovera, M., Correa, N.S., 2006. Phosphate-solubilizing *Pseudomonas putida* can influence the rhizobia-legume symbiosis. Soil Biol. Biochem. 38, 3502–3505. https://doi.org/10.1016/j.soilbio.2006.05.008.

Rubio, G., Pereyra, F.X., TAboada, M.A., 2019. The Soils of Argentina, The Soils of Argentina. Springer International Publishing. https://doi.org/10.1007/978-3-319-76853-3.

Sannazzaro, A.I., Bergottini, V.M., Paz, R.C., Castagno, L.N., Menéndez, A.B., Ruiz, O.A., Pieckenstain, F.L., Estrella, M.J., 2011. Comparative symbiotic performance of native rhizobia of the flooding Pampa and strains currently used for inoculating *Lotus tenuis* in this region. Antonie van Leeuwenhoek. Int. J. Gen. Mol. Microbiol. 99. https://doi.org/10.1007/s10482-010-9502-9.

Sannazzaro, A.I., Tejerizo, G.T., Fontana, M.F., Cumpa Velásquez, L.M., Hansen, L.H., Pistorio, M., Estrella, M.J., 2018. *Mesorhizobium sanjuanii* sp. Nov., isolated from nodules of lotus tenuis in the saline-alkaline lowlands of Flooding Pampa, Argentina. Int. J. Syst. Evol. Microbiol. 68, 2936–2942. https://doi.org/10.1099/ijsem.0.002924.

Sarkar, A., Islam, T., Biswas, G.C., Alam, S., Hossain, M., Talukder, N.M., 2012. Screening for phosphate solubilizing bacteria inhabiting the rhizoplane of rice grown in acidic soil in Bangladesh. Acta Microbiol. Immunol. Hung. 59, 199–213. https://doi.org/10.1556/AMicr.59.2012.2.5.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus 2 (1). https://doi.org/10.1186/2193-1801-2-587.

Sharma, S., Kulkarni, J., Jha, B., 2016. Halotolerant rhizobacteria promote growth and enhance salinity tolerance in peanut. Front. Microbiol. 7, 1–11. https://doi.org/10.3389/fmicb.2016.01600.

Simontacchi, M., García-Mata, C., Bartoli, C.G., Santa-María, G.E., Lamattina, L., 2013. Nitric oxide as a key component in hormone-regulated processes. Plant Cell Rep. 32, 853–866. https://doi.org/10.1007/s00299-013-1434-1.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. W. H. Freeman and Co.

Son, H.J., Park, G.T., Cha, M.S., Heo, M.S., 2006. Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. Bioresour. Technol. 97, 204–210. https://doi.org/10.1016/j.biortech.2005.02.021.

Sparks, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Sumner, M.E., Miller, W.P., 1996. Cation exchange capacity and exchange coefficients. In: Methods of Soil Analysis Part 3—Chemical Methods.

Sperry, J.F., Wilkins, T.D., 1976. Arginine, a growth limiting factor for Eubacterium lentum. J. Bacteriol. 127, 780–784. https://doi.org/10.1128/jb.127.2.780-784.1976.

Stoffella, S., Posse, G., Collantes, M., 1998. Variabilidad fenotipica y genotipica de poblaciones de *Lotus tenuis* que habitan suelos con distinto pH. Ecol. Austral 8, 57–63.

Teakle, N.L., Amtmann, A., Real, D., Colmer, T.D., 2010. Lotus tenuis tolerates combined salinity and waterlogging: maintaining O2 transport to roots and expression of an NHX1-like gene contribute to regulation of Na+ transport. Physiol. Plant. 139, 358–374. https://doi.org/10.1111/j.1399-3054.2010.01373.x.

Torbaghan, M.E., Lakzian, A., Astaraei, A.R., Fotovat, A., Besharati, H., 2017. Salt and alkali stresses reduction in wheat by plant growth promoting haloalkaliphilic bacteria. J. Soil Sci. Plant Nutr. 17, 1058–1073. https://doi.org/10.4067/S0718-95162017000400016.

Walkley, A., Black, I.A., 1934. An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci. 37, 29–37.

Zgadzaj, R., Garrido-Oter, R., Jensen, D.B., Koprivova, A., Schulze-Lefert, P., Radutoiu, S., 2016. Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. Proc. Natl. Acad. Sci. 113, E7996–E8005. https://doi.org/10.1073/pnas.1616564113.

Zheng, B.-X., Hao, X.-L., Ding, K., Zhou, G.-W., Chen, Q.-L., Zhang, J.-B., Zhu, Y.-G., 2017. Long-term nitrogen fertilization decreased the abundance of inorganic phosphate solubilizing bacteria in an alkaline soil. Sci. Rep. 7, 42284 https://doi.org/10.1038/srep42284.

Zheng, B.X., Zhang, D.P., Wang, Y., Hao, X.L., Wadaan, M.A.M., Hozzein, W.N., Peñuelas, J., Zhu, Y.G., Yang, X.R., 2019. Responses to soil pH gradients of inorganic phosphate solubilizing bacteria community. Sci. Rep. 9, 1–8. https://doi.org/10.1038/s41598-018-37003-w.