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ORIGINAL ARTICLE

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)

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Keywords

gluconic acid, *Lotus tenuis, Pantoea eucalypti,* PGPR, phosphate-solubilizing bacteria.

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Abstract

Aims: To isolate and characterize phosphate-solubilizing strains from a constrained environment such as the Salado River Basin and to assess their phosphate-solubilizing mechanisms, to further selection of the most promising strains to inoculate and improve the implantation and persistence of *Lotus tenuis* in the most important area devoted to meat-cow production in Argentina.

Methods and Results: Fifty isolates were obtained and through BOX-PCR analysis, 17 non-redundant strains were identified. Subsequently, they were found to be related to *Pantoea*, *Erwinia*, *Pseudomonas*, *Rhizobium* and *Enterobacter* genera, via 16S rRNA gene sequence analysis. This was in agreement with the clusters obtained by antibiotic resistance analysis. All isolates were tested for their phosphate-solubilizing activity and selected strains were inoculated onto *L. tenuis* plants. The most efficient isolate, was identified as *Pantoea eucalypti*, a novel species in terms of plant growth-promoting rhizobacteria.

Conclusions: The isolates obtained in this study showed a significant *in vitro* plant-growth promoting activity onto *Lotus tenuis* and the best of them solubilizes phosphate mainly via induction of the metabolism through secretion and oxidation of gluconic acid.

Singnificance and Impact of the Study: The use of these bacteria as bioinoculants, alone or in combination with nitrogen-fixing micro-organisms, could be a sustainable practice to facilitate the nutrient supply to *Lotus tenuis* plants and preventing negative side-effects such as eutrophication.

Introduction

Phosphorus (P) is one of the major essential macronutrients for biological growth and development. Most agricultural soils contain large reserves of phosphorus, which have been accumulated mostly as a consequence of regular applications of P fertilizers. However, a large portion of the soluble inorganic phosphate in fertilizers is rapidly inmobilized in the soil and becomes unavailable to plants (Rodríguez and Fraga 1999).

For over a century, agricultural microbiologists and microbial ecologists have studied the ability of some bacteria to dissolve poorly soluble calcium mineral phosphates (CaPs) (Goldstein *et al.* 1999). Micro-organisms assimilate P via membrane transport, so dissolution of CaPs to Pi ($H_2PO_4^-$ and HPO_4^{2-}) is considered to be essential to the global P cycle (Goldstein *et al.* 2003).

Phosphate-solubilizing bacteria (PSB) mobilize insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots (Sashidhar and Podile 2010). In turn, plants supply root-borne C-compounds, mainly sugars, which can be metabolized for bacterial growth (Deubel *et al.* 2000). In this sense PSB hold the potential of ecological amelioration of

degraded and disturbed forest wastelands by improving growth and establishment of plants under low P availability (Gulati *et al.* 2008).

It is frequently accepted that the main mechanism of mineral phosphate-solubilization by phosphate-solubilizing strains is associated with the release of low molecular weight organic acids (Goldstein 1995; Kim *et al.* 1998) which, via their hydroxyl and carboxyl groups, chelate the phosphate-bound cations thereby converting it into soluble forms (Vyas and Gulati 2009). Evaluation of a large number of samples from a wide range of soil types has shown that, in general, highly efficient PSB are Gram-Negative and utilize the direct oxidation glucose pathway to produce gluconic and 2-ketogluconic acids (Krishnaraj and Goldstein 2001). Conversion of glucose to gluconic acid is facilitated by pyrolloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) and gluconic acid oxidation to 2-ketogluconic takes place via the FAD-linked gluconate dehydrogenase (GADH) (Buurman *et al.* 1994; Buch *et al.* 2008). Both enzymes are in the outer face of the cytoplasmic membrane, so acids are formed in the periplasmic space, with the resultant acidification of this region and, ultimately, the adjacent medium as well (Babu-Khan *et al.* 1995).

To date, bacteria recognized as PSB belong to the genera *Pseudomonas*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, *Acinetobacter*, *Erwinia* and *Pantoea* (Rodríguez and Fraga 1999; Torres *et al.* 2008; Peix *et al.* 2009). However the phosphate solubilizing mechanism has been studied mostly in species of the genera *Pseudomonas* and *Erwinia* (Liu *et al.* 1992; Vyas and Gulati 2009).

In the Salado River Basin, an extensive area in Buenos Aires province (Argentina) mainly devoted to cattle production, the soils present a severe phosphorus deficiency (2–10 mg kg^{-1} of available phosphorus), high alkalinity and salinity levels, together with periodic exposure to waterlogging conditions, which significantly decrease persistence and yield of traditional forage legumes (i.e. lucerne, trefoil, etc.) (Montes 1988). In this region, the best-adapted species used as forage for cattle production are native grasslands and the naturalized legume, *Lotus tenuis* (= *L. glaber* Mill.) (Kirkbride 1990). *L. tenuis* is a glycophytic, perennial legume of European origin highly valuable because of its contribution to the forage offer in the region and its influence on growth of associated species (Montes 1988; Díaz *et al.* 2005).

It has been reported that phosphorus deficiency in soils may also have a deleterious effect on symbiotic interaction between rhizobia and legumes like *L. tenuis*, thus affecting its growth and productivity (Gyaneshwar *et al.* 2002). In this way, inoculation of *L. tenuis* with selected phosphate-solubilizing bacteria which are expected to increase the level of available phosphorus in soil should improve the nitrogen-fixation process of this legume and the global forage production in the Salado River Basin.

Despite the benefits that phosphate-solubilizing bacteria could represent to legume cultivation, the information about native strains with this ability from the rhizosphere of *Lotus* species in Argentinian soils, and particularly in saline-alkalyne soils of the Salado River Basin, is still very sparse. Therefore, the aims of the present study were the isolation and characterization of phosphate-solubilizing strains from this environments and the assessment of their phosphate-solubilizing mechanisms, to further selection of the most promising strains to inoculate and improve the implantation and the persistence of *L. tenuis* in constrained environments such as the Salado River Basin.

Methods

Soil samples and isolation of phosphate-solubilizing bacteria

Soil samples from the rhizospfere of *Lotus tenuis* in lowlands of the Salado River Basin were taken at several sites located in Chascomús County (Latitude 35°30′S. Longitude 58°30′W), in Buenos Aires Province (Argentina). Thirteen different samples with a phosphorus content lower than 3 mg kg^{-1} and pH values between 6·7 and 9·7 were collected in the field, transported to the laboratory and stored at 4°C for 2 days prior to use.

For isolation of phosphate-solubilizing bacteria, several sub-samples from each soil sample were homogenized in sterile distilled water and serially diluted. Aliquots of each dilution were spread on NBRIP medium (5 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0·25 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0·2 g l^{-1} KCl, 0·1 g l^{-1} $(NH_4)_2SO_4$, 5 g l^{-1} $Ca_3(PO_4)_2$ and 10 g l^{-1} glucose) (Nautiyal 1999) and incubated at 30°C for 24–48 h. Colonies were selected on the basis of the development of a clear halo; the clones were further purified on NBRIP and TY (Sperry and Wilkins 1976) media. Once purified, each isolate was stored at −80°C in the same medium with 20% (v/v) glycerol.

In order to describe the phenotypic characteristics of the isolates, parameters such as shape (punctiform, circular, rhizoid, irregular or filamentous), colour, edge (entire, undulate, lobate, filamentous or curled), surface (smooth, glistening, rough, wrinkled, dry or powdery), and elevation (flat, raised, convex, pulvinate or umbonate) were recorded for each isolate growing on TY medium.

Antibiotic susceptibility analysis

The antibiotic susceptibility of each isolate was determined according to the guidelines of the National Committee for

Clinical Laboratory Standards (Bauer *et al.* 1966), using antibiotic sensitivity BGN 1 disc rings from Britania™.

Siderophore and phytohormones production

Siderophore production of each isolate was determined on Chrome-azurol S (CAS) medium following the Universal Chemical Assay (Schwyn and Neilands 1987). Bacterial strains (24-h-old cultures) were spotted on CAS plates and incubated at 28 ± 1°C for 48 h. The production of siderophores was indicated by the formation of orange to yellow halo around the colonies. The Indole Acetic Acid (IAA) and Zeatine (Z) levels were determined as previously described by Perrig *et al.* (2007).

Mineral phosphate-solubilization assays

The solubilization efficiency of each isolate was determined through the kinetics of $Ca_3(PO_4)_2$ solubilization in NBRIP liquid medium at 24, 48 and 72 h of incubation. Tubes (20 ml), containing 4 ml of medium inoculated with each isolate (40 μl inoculum with approximately 1×10^9 CFU ml^{-1}), were incubated at 30°C and 180 rev min^{-1}. The cultures were harvested by centrifugation at 13 500 ***g*** for 5 min and inorganic phosphate concentration was determined colorimetrically in the supernatant by the molybdenum-blue method (Murphy and Riley 1962). Pellet was further processed to determine protein concentration according to Bradford (1976) and phosphate-solubilization was referred to protein content.

Assessment of the effect of soluble phosphate on glucose dehydrogenase and gluconate-2-dehydrogenase enzymatic activities and gluconic acid release

The isolates were grown in NBRIP medium amended with different soluble phosphate levels (K_2HPO_4) from 0 to 50 mmol l^{-1}. Aliquots of bacterial culture were collected at several times after inoculation and centrifuged at 9200 ***g*** for 3 min at 4°C. Supernatant was used for pH, solubilized phosphate and gluconic acid determinations. Enzymatic assays were performed with whole cells suspensions, which were harvested and washed twice with sterile saline water and resuspended in 20 mmol l^{-1} Tris–HCl buffer, pH 7·5, containing 3 mmol l^{-1} $CaCl_2$.

Glucose dehydrogenase (GDH) and gluconate-2-dehydrogenase (GADH) enzyme activities were determined spectrophotometrically at 25°C by following the reduction of 2,6-dichlorophenol-indophenol (DCIP) at 600 nm, using phenazine methosulfate (PMS) as a primary electron acceptor and according to the methods described by Olsthoorn and Duine (1996), and Matsushita *et al.* (1982) respectively.

The amount of gluconic acid produced in culture filtrates was measured with a commercial enzyme kit for the determination of D-gluconic acid/D-glucono-δ-lactone (RBiopharm, Darmstadt, Germany).

Molecular identification: BOX-PCR amplification and sequencing of 16S rRNA gene

Extraction of DNA from isolates for PCR amplifications
All the isolated strains were grown for 48 h at 28°C and a loopful of young cells from each isolate was suspended in 0·1 ml of MilliQ water. For BOX-PCR reactions, the cell suspensions were centrifuged at 13 500 ***g*** for 5 min and the supernatant was removed. The pellet was resuspended in 0·1 ml of sterile MilliQ water and cells were lysed by boiling for 15 min and then centrifuged at 13 500 ***g*** for 5 min. Finally, the supernatant was transferred to a clean tube and used as DNA source (Sannazzaro *et al.* 2010). For 16S sequence reactions, total DNA was extracted from 3-ml cultures of each bacterial isolate according to the method described by Estrella *et al.* (2009).

BOX-PCR
BOX-PCR (Versalovic *et al.* 1994) was used to assess the genetic diversity of the isolates. PCR assays were performed using the universal BOXA1R primer (5′CTACGGCAAGGCGACGCTGACG 3′; Versalovic *et al.* 1994) synthesized by Ruralex Fagos, Argentina. PCR amplification was carried out in a 25 μl reaction mixture containing 2 μmol l^{-1} BOX A1R primer, 1·25 mmol l^{-1} each deoxynucleoside triphosphate (Promega, Madison, WI), 1 × polymerase reaction buffer (PB-L, Bernal, Bs As, Argentina), 7 mmol l^{-1} $MgCl_2$ (PB-L, Argentina), 1 U Taq Pegasus DNA polymerase (PB-L, Argentina) and 6 μl of DNA. Amplification was performed with a Px2 thermal cycler (Thermo Electron Corp., Waltham, MA) using the following temperature profile: initial denaturation at 95°C for 6 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and elongation at 65°C for 8 min; and a final extension of 16 min at 65°C (Versalovic *et al.* 1994). Amplified fragments were separated by electrophoresis in 1·5% agarose gels for 2 h at 85 V in 1 × Tris–borate–EDTA buffer (100 mmol l^{-1} Tris, 83 mmol l^{-1} boric acid, 1 mmol l^{-1} EDTA; pH 8·5). A 100-bp DNA ladder (PB-L, Argentina) was used as a molecular weight marker. Gels were stained in an aqueous solution containing ethidium bromide (0·1%) and photographed using a UV transilluminator.

16S rRNA
The gene-encoding 16S rRNA was amplified from selected strains by PCR using the bacterial universal primers 41f (5′-GCTCAAGATTGAACGCTGGCG-3′) and 1488r

(5′-CGGTTACCTTGTTACGACTTCACC-3′) as previously described (Estrella *et al.* 2009).

The PCR products were purified using the GFX kit (GE Healthcare, Little Chalfont Buckinghamshire, UK) and sequenced using an Applied Biosystems ABI 377 sequencer (DNA Sequencing Service, Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín – UNSAM, Argentina). The obtained sequences were compared with the sequences of reference strains deposited in the GenBank (Table 1) using the Blastn program (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis

Sequence alignment was performed with the ClustalW software from the EMBL server (http://www.ebi.ac.uk/). Aligned sequences were analysed using the mega

Table 1 Reference strains used in this study

Strain	Species	16S rRNA accession no.	Reference
ATCC 29283	*Erwinia rhapontici*	U80206.1	(Kwon *et al.* 1997)
LMG 2688	*Erwinia rhapontici*	Z96087.1	(Hauben *et al.* 1998)
DSM 4484	*Erwinia rhapontici*	AJ233417.1	(Sproer *et al.* 1999)
LMG 11254	*Erwinia persicinus*	Z96086.1	(Hauben *et al.* 1998)
ATCC 15580	*Erwinia amylovora*	U80195.1	(Kwon *et al.* 1997)
Et1/99	*Erwinia tasmaniensis*	AM055716.1	(Geider *et al.* 2006)
ATCC 29267	*Erwinia cypripedii*	U80201.1	(Kwon *et al.* 1997)
DSM 30104	*Klebsiella pneumoniae*	AJ233420.1	(Sproer *et al.* 1999)
JCM 1662	*Klebsiella pneumoniae*	AB004753.1	(Harada *et al.* 1996)
Ola 50	*Enterobacter oryzae*	EF488759.1	(Peng *et al.* 2009)
Ola 1	*Enterobacter oryzae*	EF488760.1	(Peng *et al.* 2009)
LMG 2715	*Pantoea stewartii*	Z96080.1	(Hauben *et al.* 1998)
ATCC 19321	*Pantoea ananatis*	U80209.1	(Kwon *et al.* 1997)
LMG 2676	*Pantoea ananatis*	FJ611846.1	(Rezzonico *et al.* 2009)
LMG 5342	*Pantoea ananatis*	FJ611845.1	(Rezzonico *et al.* 2009)
ATCC 33244	*Pantoea ananatis*	NR_026045.1	(Kwon *et al.* 1997)
LMG 24200	*Pantoea deleyi*	EF688011.1	(Brady *et al.* 2008)
ATCC 43348	*Pantoea agglomerans*	FJ611821.1	(Rezzonico *et al.* 2009)
DSM 3493	*Pantoea agglomerans*	AJ233423.1	(Sproer *et al.* 1999)
ATCC 27987	*Pantoea agglomerans*	FJ611824.1	(Rezzonico *et al.* 2009)
LMG 24199	*Pantoea vagans*	EF688012.1	(Brady *et al.* 2008)
LMG 24197	*Pantoea eucalypti*	EF688009.1	(Brady *et al.* 2008)
Pf-5	*Pseudomonas fluorescens*	CP000076.1	(Paulsen *et al.* 2005)
ICMP 3512	*Pseudomonas fluorescens*	AJ308308.1	(Hilario *et al.* 2004)
CCM 2115	*Pseudomonas fluorescens*	DQ207731.2	(Sipos *et al.* 2007)
IAM 1236	*Pseudomonas putida*	D84020.1	(Anzai *et al.* 1997)
ATCC 12633	*Pseudomonas putida*	AJ308313.1	(Hilario *et al.* 2004)
KT 2440	*Pseudomonas putida*	NC_002947.3	(Nelson *et al.* 2002)
DSM 291	*Pseudomonas putida*	Z76667.1	(Moore *et al.* 1996)
ATCC 23344	*Burkholderia mallei*	CP000011.2	(Nierman *et al.* 2004)
MTI-641	*Burkholderia unamae*	AY221956.1	(Caballero-Mellado *et al.* 2004)
A-1Bs	*Mesorhizobium tianshanense*	AF041447.1	(Wang *et al.* 1999)
IFO 15243	*Mesorhizobium huakuii*	D13431.1	(Oyaizu *et al.* 1993)
NZP 2213	*Mesorhizobium loti*	D14514.1	(Laguerre *et al.* 1993)
CCBAU 21244	*Rhizobium* sp.	AY555768.2	(Liu *et al.* 2005)
MTR 35B	*Agrobacterium* sp.	DQ507210.1	(Grandlic *et al.* 2008)
CIAT 899	*Rhizobium tropici*	U89832.1	(Van Berkum *et al.* 1996)
DSM 30105	*Agrobacterium tumefaciens*	M11223.1	(Yang *et al.* 1985)
ICMP 12856	*Agrobacterium rhizogenes*	AY626393.1	(Young *et al.* 2004)
CFN 42	*Rhizobium etli*	U28916.1	(Van Berkum *et al.* 1996)
3841	*Rhizobium leguminosarum* bv. *viciae*	AM236085.1	(Young *et al.* 2006)
R 602	*Rhizobium gallicum*	AF008130.1	(Sessitsch *et al.* 1997)
BD 60	*Rhizobium* sp.	EU748909.1	(Estrella *et al.* 2009)
ML 92	*Rhizobium* sp.	EU748919.1	(Estrella *et al.* 2009)
ML 98	*Rhizobium* sp.	EU748921.1	(Estrella *et al.* 2009)

software, version 4·0 (Tamura *et al.* 2007). Phylogenetic analyses of the 16S rRNA sequences were performed by the UPGMA method (Sneath and Sokal 1973). The phylogenetic distances were computed by the p-distance method and calculated based on the proportion of different nucleotides (p-distance), which was obtained by dividing the number of nucleotide differences by the total number of nucleotides compared (Nei and Kumar 2000). Statistical support for tree nodes was evaluated by bootstrap analysis (Felsenstein 1985).

Accession numbers

The nucleotide sequence data reported in this paper appear with the following accession numbers (GenBank: from HM008943 to HM008959).

In vitro PGPR activity assay

Plant material and growth conditions

Lotus tenuis seeds were scarified and surface disinfected as previously described by Estrella *et al.* (2009) and germinated in the dark for 24–48 h at 25°C. Seedlings were transferred to tubes (2·5 cm diameter, 20 cm height) or flasks (6 cm diameter, 10 cm height) containing 5 or 75 ml of semisolid modified Evans medium, respectively (Evans *et al.* 1970). Soil stress conditions were simulated by replacing the soluble phosphate source with 100 mg l^{-1} of $Ca_3(PO_4)_2$ and varying P/N ratio using 10 and 1 mg l^{-1} N (as NH_4NO_3). Plants were grown in a greenhouse with a 16/8 h photoperiod at 25°C/21°C (day/night) and 55/75 ± 5% r.h.. Light intensity (200 μmol m^{-2} s^{-1}) was provided by incandescent and cool white fluorescent lamps.

Five-day-old *L. tenuis* seedlings growing aseptically in flasks were inoculated with a bacterial suspension (10^8 cells per seedling) in a completely randomized design (five replicate flasks per treatment containing four plants each). Non-inoculated plants were used as negative controls. At harvest (45 days post-inoculation) the dry weight (DW) of the aerial part of the plants was determined. Plant phosphorus content was measured by the molybdenum-blue method after acid digestion of the plant material (Murphy and Riley 1962).

Statistical analysis

Data from greenhouse experiments were analysed by two-way analysis of variance (two-way ANOVA), followed by all pairwise multiple comparisons (*post hoc* testing), using the Tukey method at $P < 0{\cdot}001$. Pearson's correlation coefficients between solubilized phosphate and pH of the medium were calculated. Data from enzymatic activity determination and gluconic acid content were analysed by one-way-analysis of variance (one-way ANOVA), followed by all pairwise multiple comparisons (*post hoc* testing), using the Tukey and Holm–Sidak methods at $P < 0{\cdot}001$, respectively.

Results

Isolation and identification of phosphate-solubilizing bacteria from soil samples

Isolation

Isolation of phosphate-solubilizing bacteria colonies was achieved in NBRIP medium. Fifty isolates with this ability were obtained from 10 soil samples with pH values between 6·7 and 8·1. On the contrary, no isolates were obtained from three soil samples with pH values of 8·9, 9·5 and 9·7 respectively. The isolates were selected according to differences in morphology and $Ca_3(PO_4)_2$ solubilization halos.

BOX fingerprinting analysis

In order to identify non-redundant strains, BOX-PCR fingerprinting of each isolate was performed. From the initial 50 isolates, several of them presented identical profiles but some isolates with a unique and complex fingerprint were also found (data not shown). Through this analysis, 17 non-redundant strains were identified and used for further analysis.

Phenotypical characterization, antibiotic resistance and siderophore production

Phenotypic characteristics of the 17 isolates with non-redundant fingerprintings were assessed by describing the shape, colour, edges, surface and elevation of the whole colony on TY medium and are summarized in Table 2. Antibiotic resistance was also determined for the 17 strains (Table 2). A wide antibiotic resistance range was found in five of the total isolates (I26, M22, M25, M51 and M77), while susceptibility to all the tested antibiotics was observed in the remaining 12 isolates. Noteworthy, siderophore production was observed in all isolates, this being an important feature for a strain to be considered as a plant growth promoting rhizobacteria (PGPR) (Podile and Kishore 2006; Ahmad *et al.* 2008).

Analysis of 16S rRNA gene sequences

Nearly full-length 16S rRNA sequences were obtained from the 17 isolates with non-redundant BOX-profiles and sequence analyses of 16S rRNA genes were performed in order to identify their phylogenetic position. Figure 1 shows a phylogenetic tree based on the similarity of the 16S rRNA sequences of the isolates and reference strains,

Table 2 Phenotypic characteristics and antibiotic resistance traits of the selected isolates

Isolate	Morphology*	Antibiotic phenotype†					
		IMP	GEN	CAZ	AMS	CTX	TMS
I17	Circular white entire smooth raised	S	S	S	S	S	S
I26	Irregular yellow entire smooth raised	I	S	S	R	R	R
I29	Circular opac-white entire smooth raised	S	S	S	S	S	S
I35	Circular white entire smooth raised	S	S	S	S	S	S
I38	Circular white entire smooth raised	S	S	S	S	S	S
M22	Irregular yellow undulate smooth raised	S	S	S	R	R	R
M25	Irregular yellow lobate wrinkled flat	R	I	R	R	R	R
M51	Circular beige entire smooth raised	S	R	R	I	R	S
M52	Circular beige entire smooth raised	S	S	S	S	S	S
M56	Irregular yellow undulate smooth raised	S	S	S	I	S	S
M75	Circular yellow entire smooth raised	S	S	S	S	S	S
M76	Irregular white undulate glistening convex	S	S	S	S	S	S
M77	Irregular beige undulate smooth raised	S	S	R	I	I	S
M78	Circular yellow entire smooth raised	S	S	S	S	S	S
M87	Irregular pale-yellow entire smooth raised	S	S	S	S	S	S
M89	Circular white entire smooth raised	S	S	S	S	S	S
M91	Irregular yellow undulate smooth raised	S	S	S	S	S	S

Assayed antibiotics. IMP: imipenem, GEN: gentamicin, CAZ: ceftazidime, AMS: ampicillin/sulbactam, CTX: cefotaxime, TMS: trimethoprim/sulfamethoxazole.

*Colony morphology (whole colony-color-edge-surface-elevation) in TY medium.

†S, R and I mean susceptibility, resistance or intermediate resistance, respectively.

which was constructed using the p-distance model for estimating phylogenetic distances (Nei and Kumar 2000) and the UPGMA algorithm (Sneath and Sokal 1973).

On the basis of 16S sequences, the isolates grouped with species belonging to five genera, all of which are known to contain phosphate-solubilizing bacterial species. Thus, isolates I35, I38, I26, M52 and I17 grouped with the genus *Erwinia* and clustered in a separated branch together with strains of *Erw. rhaponticci* species, a pathogen of pea *Pisum sativum* L. (Schroeder *et al.* 2002). The isolates displayed 99% sequence identity with *Erw. rhaponticci* ATCC 29283 (Fig. 1).

Isolates M76 and M87 were closely related to species of the genus *Enterobacter*, sharing respectively 98 and 97% sequence identity with *Ent. oryzae Ola 1* and *Ent. oryzae Ola 50*; both endophytic strains isolated from wild rice species *Oryza talifolia* (Fig. 1) (Peng *et al.* 2009).

Isolates M91, M56, M78 and M89 grouped with the *Pantoea* cluster but branched out of the *Pa. agglomerans–Pa. vagans* cluster. These isolates formed a differentiated branch together with *Pa. eucalypti* LMG 24197, isolated from eucalyptus leaves and shoots (Brady *et al.* 2009). The level of 16S rRNA sequence identity between this reference strain and the isolates above mentioned was 99% (Fig. 1).

According to 16S rRNA sequences, the isolates M25, M22, M77 and M75 grouped with the genus *Pseudomonas*. M25 was related to *Ps. fluorescens* species showing 99% sequence similarity with *Ps. fluorescens* Pf-5, a plant commensal bacterium with suppressor activities for plant pathogens (Fig. 1) (Paulsen *et al.* 2005). On the other hand, isolates M22, M77 and M75 were closely related to *Ps. putida cluster*, with values of sequence identity between 98 and 99% with reference strains of this cluster (Fig. 1).

In the case of isolates M51 and I29, both of them grouped with species related to plant-related bacteria. The isolate M51 shared 99% of sequence similarity with *Agrobacterium* sp. *MTR* 35B, a plant growth-promoting bacterium (Grandlic *et al.* 2008), and 99% with *Rhizobium* sp. *CCBAU 21244,* a root nodule bacteria isolated from leguminous trees in China (Liu *et al.* 2005).

Isolate I29 formed a separated cluster together with strains BD 60, ML 92 and ML 98 belonging to the genus *Rhizobium,* which were isolated from nodules of *L. tenuis* and were described in a previous taxonomical study of characterization of rhizobial isolates carried out in soils of the Salado River Basin (Estrella *et al.* 2009).

Characterization of phosphate-solubilization pathway

Phosphate-solubilization kinetics

The kinetic of phosphate-solubilization of the above mentioned 17 isolates was studied by measuring the phosphate solubilized at 24, 48 and 72 h of incubation (Fig. 2a). The highest phosphate-solubilization activities

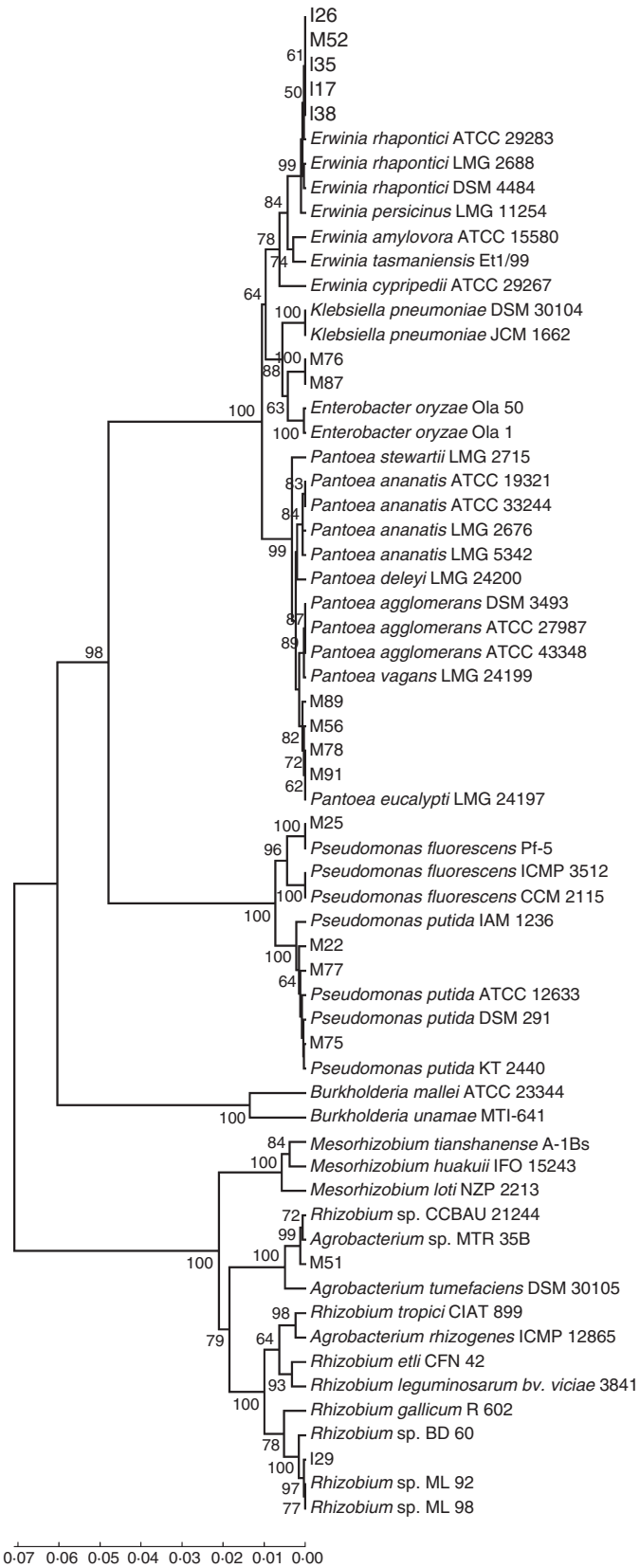

Figure 1 16S rRNA gene phylogeny of the phosphate-solubilizing bacteria isolated from *L. tenuis* rhizosphere in the Salado River Basin. The tree was constructed from the nucleotide sequence data using the UPGMA algorithm, and phylogenetic distances were calculated by the p-distance method. The numbers at branch points are the significant bootstrap values (expressed as percentages based on 1000 replicates; only values greater than 50% are shown). The horizontal branch lines are proportional and indicate the p-distances.

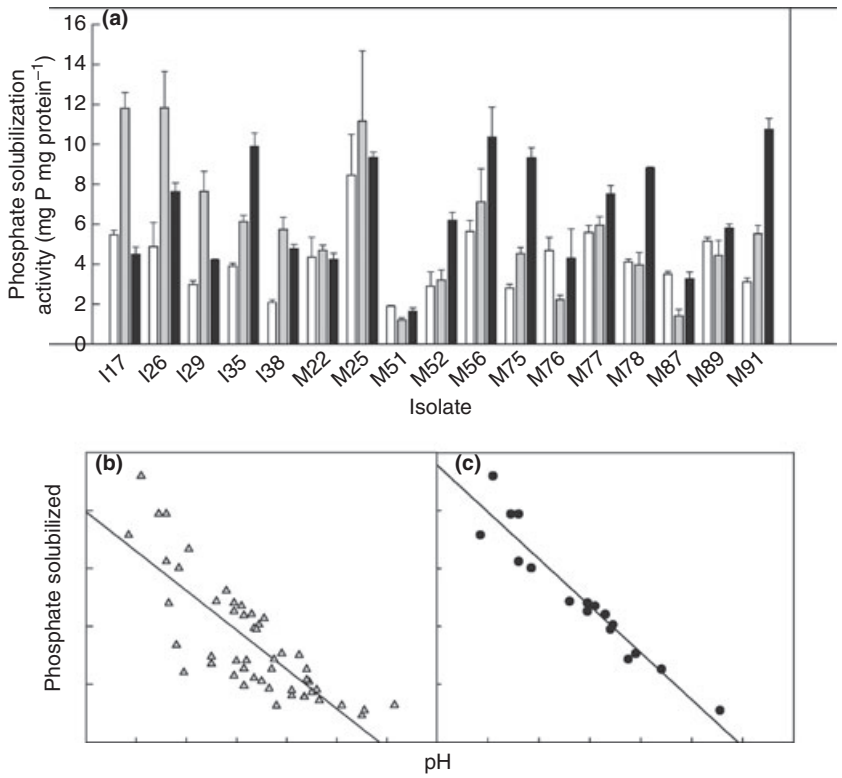

Figure 2 (a) Phosphate solubilization activity of the different isolates (mg P mg protein^{-1}). Samples were taken at different time intervals (24, 48 and 72 h) and plotted against time. Data represent mean ± SD (n = 3), and correlation between pH and solubilized phosphate in the medium (b) taking into account all the isolates at □ 24, ▒ 48 and ■ 72 h incubation (−0·753) and (c) 72 h incubation (−0·956).

were achieved by isolates M91, M25, M56, M75, M78, I35, I17 and I26. Three different patterns were observed among the selected isolates: in one group of strains a gradual activity increment was found; in a second one, the solubilization activity reached a maximum peak (at 48 h p.i.) and was then reduced, and in a third group the activity remained constant throughout the test.

On the other hand, a highly significant correlation was found between the amount of solubilized phosphate and the pH of the culture media (r = −0·753) (Fig. 2b), at 72 h. the correlation value is higher (r = −0·956) (Fig. 2c). This observation strongly suggests that the main mechanism of phosphate-solubilization would be achieved by medium acidification.

Effect of soluble phosphate on glucose dehydrogenase and gluconate-2-dehydrogenase activities and gluconic acid release

Isolate M91 was selected for these experiments given that it was one of the strains that showed a high phosphate solubilizing activity and for being identified as *Pantoea eucalypti*, a novel species in terms of plant growth promoting rhizobacteria.

Under soluble phosphate starvation, isolate M91 was found to produce gluconic acid being this production responsible for medium acidification. Gluconic acid secretion reaches a threshold at 24 h p.i. and decreases at 72 h p.i. (Fig. 3a). This pattern has a negative correlation with the amount of solubilized phosphate in the medium (Fig. 3d). Conversely, GDH activity is kept constant through time (Fig. 3b).

As a consequence of these results, GADH activity was determined to study gluconic acid pathway and this enzymatic activity was found to followed the same pattern as gluconic acid release and also to have a negative correlation with the amount of solubilized phosphate in the medium (Fig. 3c).

When the effect of soluble phosphate was studied we found that gluconic acid release was significantly higher when M91 was grown at 0 mmol l^{-1} K_2HPO_4, compared to 50 mmol l^{-1} K_2HPO_4 (Fig. 3a). Those differences were also found with both activities, GDH and GADH (Fig. 3b,c).

It is also worth pointing out that when the medium was supplemented with K_2HPO_4 (10–50 mmol l^{-1}), GDH and GADH activities did not have any changes through time (Fig. 3b,c).

Greenhouse experiments

The ability of phosphate-solubilizing bacteria to enhance the growth and phosphorus uptake of *Lotus tenuis* was studied, under controlled conditions, for a group of isolates with high *in vitro* phosphate-solubilization activity

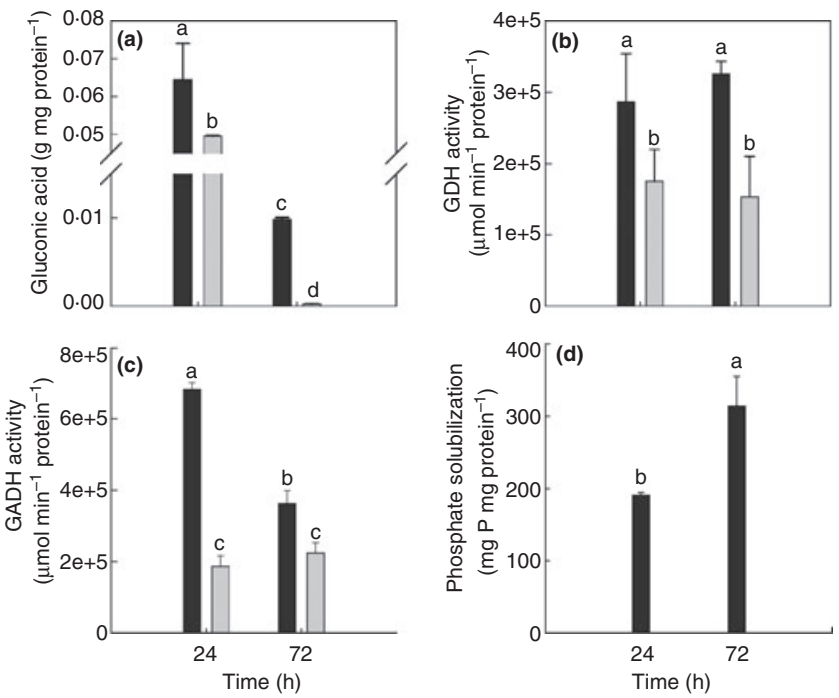

Figure 3 Gluconic acid metabolism under different soluble phosphate levels. Isolate M91 was grown in NBRIP medium (black bars) and in NBRIP medium amended with 50 mmol l^{-1} K_2HPO_4 (grey bars). Several samples were taken at 24 and 72 h of incubation for the determination of (a) gluconic acid release content; (b) glucose dehydrogenase (GDH) activity; (c) gluconate-2-dehydrogenase (GADH) activity and (d) solubilized phosphate content. Different letters indicate significant difference according to Holm–Sidak's (gluconic acid content) and Tukey's (enzymatic activities) method at $P < 0{\cdot}001$ (*post hoc* testing). Data represent mean ± SD ($n = 3$).

(Fig. 2a). These bacteria were previously identified as *Pantoea eucalypti* (M91), *Pseudomonas fluorescens* (M25) and *Erwinia rhapontici* (M52). A significant increase of plant height and dry weight was observed with all the tested isolates compared with non-inoculated plants (Suppl. Figs S1 and S2). On the basis of the high significance and reproducibility of the results obtained with M91, this isolate was chosen as the inoculant strain for further experiments. *L. tenuis* cv Pampa INTA seedlings were used to test the effect of this isolate in semisolid Evans medium harbouring different N/P ratios and pH values.

A first experiment was carried out to assess the effect of two different N concentrations on the PGPR activity of the isolate at neutral pH (Fig. 4a). At 10 mg l^{-1} N, the DW of inoculated plants was increased by 55% compared to the non-inoculated control. Moreover, the total P level per plant was higher in the inoculated ones (Fig. 4b). A similar effect was observed (32% DW increment) when the semisolid medium N content was reduced to 1 mg l^{-1}. However, the plant growth promoting effect achieved by M91 was superior in the presence of 10 mg l^{-1} N in the medium compared with 1 mg l^{-1} (Fig. 4a). These results suggest that the growth-promoting activity of this isolate depends on the P/N ratio.

A second experiment was performed in order to study the influence of pH value on the PGPR activity of the isolate M91, at 10 mg l^{-1} N. At pH 8, the DW of inoculated *L. tenuis* plants was also increased (48%) compared with non-inoculated plants, as previously noted at pH 7 (Fig. 5a). In contrast, no *L. tenuis* growth promotion was observed at pH 9, when inoculated plants were compared with non-inoculated plants (Fig. 5a).

The effect of two different N concentrations on the PGPR activity of the isolate M91 was also evaluated at pH 8 and a similar dependence on the P/N ratio was found (Fig. 5b).

On the other hand, *Lotus tenuis* growth-promoting activity was not related to phytohormone production, since no detectable levels of indolacetic acid and zeatine were found in the isolate M91 culture media (data not shown).

It is also worth pointing out that similar results were obtained with other two *L. tenuis* commercial cultivars (*L. tenuis* cv Esmeralda and *L. tenuis* cv GAPP) despite the great variability among them previously described by Kade *et al.* (2003).

Discussion

To our knowledge, this is the first study focused on the isolation and characterization of phosphate-solubilizing bacteria from the rhizosphere of *Lotus tenuis* and in introducing the species *Pantoea eucalypti* as a plant growth promoting rhizobacteria.

Fifty isolates were obtained and through BOX-PCR analysis 17 non-redundant isolates were identified. These results, together with the antibiotic resistance and morphological characterization of colonies, suggested the

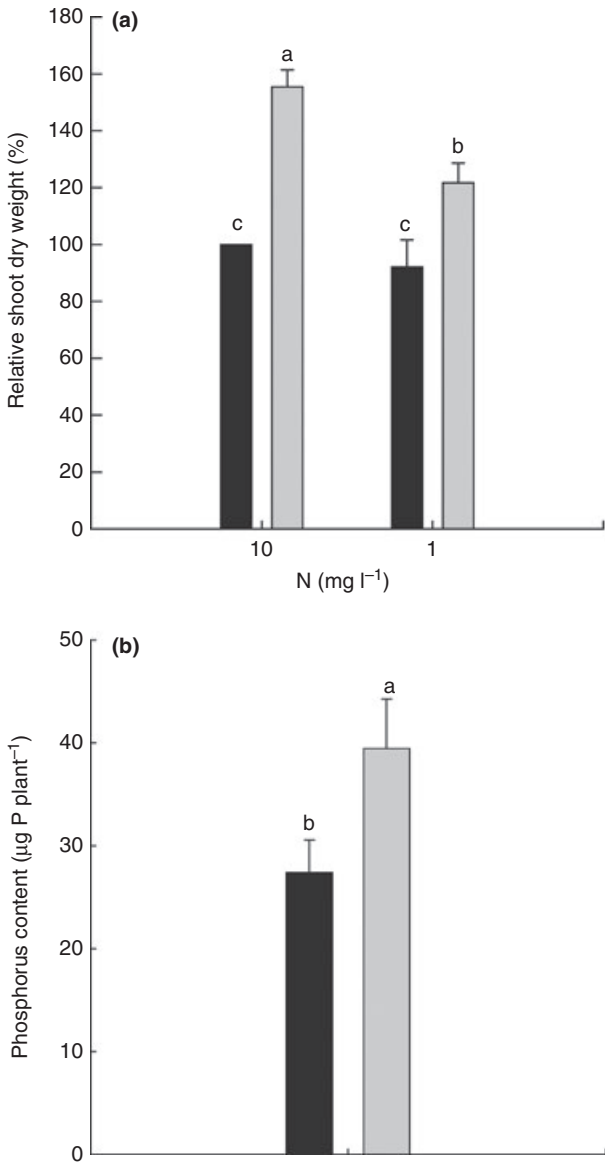

Figure 4 Effect of inoculation with isolate M91 on *L. tenuis* growth and phosphorus content at pH 7. Seedlings were inoculated in semisolid Evans medium, under phosphate deficiency. (a) Relative shoot dry weight (%) of inoculated plants (grey bars) compared with non-inoculated ones (black bars) at two N levels (1 and 10 mg l^{-1}); (b) phosphorus content in plant of inoculated plants (grey bars) compared with non-inoculated ones (black bars) at 10 mg l^{-1} N. Different letters indicate significant difference according to Tukey's method at $P < 0{\cdot}001$ (*post hoc* testing). Data represent mean ± SD ($n = 5$, i.e. five replicate flasks per treatment containing four plants each).

existence of genotypic and phenotypic diversity among the isolates; but this biodiversity is not as pronounced as it is in the case of nitrogen-fixing bacteria associated with *Lotus tenuis* (Estrella *et al.* 2009). Subsequently, the identification of isolates at the genus and species level was successfully achieved by the analysis of 16S rRNA gene sequences. The 17 phosphate-solubilizing bacteria isolated from *L. tenuis* rhizosphere were related to five genera, which have been previously reported by several authors as common phosphate-solubilizers of different mineral phosphates and isolated from the rhizosphere of legumes (Rodríguez and Fraga 1999; Kuklinsky-Sobral *et al.* 2004; Son *et al.* 2006; Sridevi and Mallaiah 2009; Sashidhar and Podile 2010). However, it is important to note that almost 25% of the isolates grouped with a cluster formed by *Pantoea eucalypti* and *Pa. vagans*, novel species closely related to *Pa. agglomerans*; which are generally considered non-pathogenic due to their lack of virulence determinants such as type III secretion systems (T3SS) (Rezzonico *et al.* 2009).

The highest phosphate-solubilization activities were achieved only by strains belonging to genera *Pantoea, Pseudomonas* and *Erwinia*. Isolates from these genera caused a significant increase in plant height and dry weight of *L. tenuis* cv Pampa INTA plants. The growth promotion resulting from the inoculation with *Pa. eucalypti* strain M91 was dependent on the pH value and the N/P ratio on the media and apparently had no dependency on the indolacetic acid and zeatine production. These results confirm the potential use of these phosphate-solubilizing bacteria as inoculants. On the other hand, the isolates M51 and I29, related to the genus *Rhizobium*, showed a low ability to solubilize phosphate. It is worth mentioning that the three rhizobial strains that grouped with isolate I29 (see Fig. 1), are *L. tenuis* symbionts isolated from soils of the Salado River Basin (Estrella *et al.* 2009), and were found to have poor phosphate-solubilization activity and low nitrogen-fixation efficiencies (unpublished results). These observations suggest that it would be useful to test inoculant formulations based on rhizobial strains in combination with phosphate-solubilizing bacteria as a more effective strategy, compared to the inoculation with one strain carrying both features, when the productivity of *L. tenuis* needs to be increased in constrained environments such as the aforementioned.

In addition, mycorhizal fungi like *Glomus intraradices* were found to be well adapted to saline-alkaline soils of the Salado River Basin, in association with roots of *L. tenuis* (Sannazzaro *et al.* 2004; Echeverria *et al.* 2008). The mycorhizal associations contribute in mobilizing phosphate from distant sources, which cannot be reached by the plant roots. However, the insoluble phosphate present in soils needs to be previously converted to its soluble forms in order to be mobilized by mycorhizal hyphae. According to this idea, Toro *et al.* (1997) suggested that these mycorhizosphere interactions between bacterial and fungal plant associates, contribute to the biogeochemical P cycling, thus promoting a sustainable

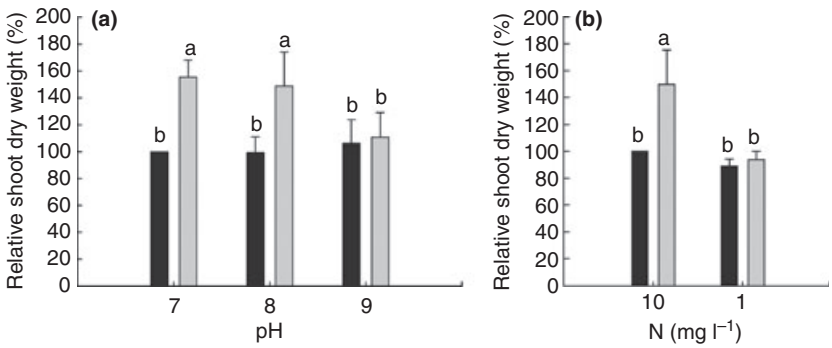

Figure 5 Effect of inoculation with isolate M91 on *L. tenuis* growth at different pH levels. Seedlings were inoculated in semisolid Evans medium, under phosphate deficiency. (a) Relative shoot dry weight (%) of inoculated plants (grey bars) compared with non-inoculated ones (black bars) at pH 7, 8 and 9; (b) relative shoot dry weight (%) of inoculated plants (grey bars) compared with non-inoculated ones (black bars) at pH 8 under two N levels (1 and 10 mg l^{-1}). Different letters indicate significant difference according to Tukey method at $P < 0{\cdot}001$ (*post hoc* testing). Data represent mean ± SD ($n = 5$, i.e. five replicate flasks per treatment containing four plants each).

nutrient supply to plants. In this regard, the phosphate-solubilizing bacteria isolated from the Salado River Basin could play a relevant role in the mycorhizal symbiosis and, in consequence, in the *L. tenuis* productivity.

It is also worth mentioning that all phosphate-solubilizing bacteria selected in this work were found to produce siderophores on CAS medium. This feature has been related to improve plant iron nutrition (Podile and Kishore 2006) and to biocontrol capacity (Khan *et al.* 2006), therefore could provide an advantageous mechanism of adaptation for both, plant and bacteria, to the edaphic conditions of the Salado River Basin.

Regarding the phosphate-solubilization mechanism, a positive correlation between the acidification of NBRIP liquid medium and the amount of solubilized phosphate was found among all the isolates, suggesting that organic acid release may play a significant role in phosphate-solubilization. A similar relationship was reported by Illmer and Schinner (1995) and Hwangbo *et al.* (2003). In gram-negative bacteria this acidification is attributed to the presence of gluconic acid. In order to confirm this relationship under different phosphate levels, the enzymes involved in gluconic acid metabolism and the gluconic levels were studied. The results indicated that the release of gluconic acid depends on the soluble phosphate level of the medium, and this was confirmed by the differences on gluconic levels when M91 was grown at 0 and 10–50 mmol l^{-1} K_2HPO_4. This is also evident when, at 72 h p.i., initially growing cells without soluble phosphate reaches a sufficient amount of it.

The results obtained with GDH activity correlated with the differences seen under different K_2HPO_4 concentrations. However, this activity remained constant throughout the experiments (72 h), thus suggesting that gluconic acid production does not change. In addition to this, the enzyme that oxidizes gluconic acid (GADH) followed the same pattern as the acid release. All these data are in agreement with Fuhrer *et al.* (2005) and Bernardelli *et al.* (2001) who have pointed out that the expression of an active GDH provides energetic and competitive advantages to different organisms, under certain environmental conditions; being this an ancillary energy-generating system. Our results also suggest that under soluble phosphate starvation gluconic acid is released to the medium and/or converted to 2-ketogluconic acid (pK_a: 2·66) which has more phosphate-solubilization ability than gluconic acid (pK_a: 3·41) (Kim *et al.* 2003). Conversely, when soluble phosphate level is appropriated, gluconic acid could be phosphorylated and metabolized via Entner–Doudoroff pathway.

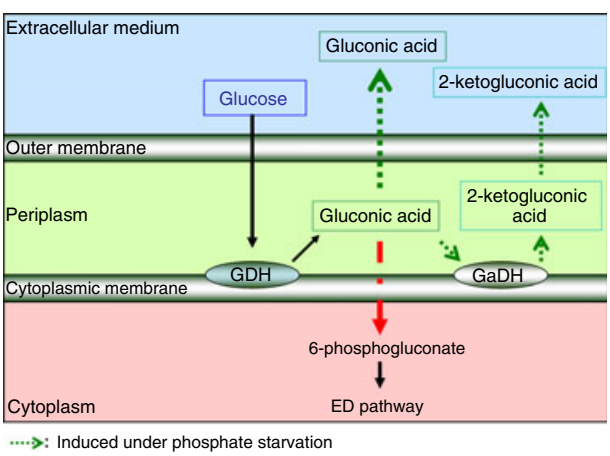

Figure 6 Schematic pathway for phosphate-solubilization in *Pantoea eucalypti*. Isolate M91 solubilizes phosphate mainly via induction of the metabolism through the secretion and oxidation of gluconic acid.

In conclusion, the combination of a biotechnological approach (microbial inoculation) with a low-input technology could be a sustainable practice to facilitate the nutrient supply to plants. In order to achieve this biotechnological approach genetically diverse phosphate-solubilizing bacteria from *Lotus tenuis* rhizosphere were isolated. Some of the isolates showed a significant plant growth promoting activity onto this legume and the best of them solubilizes phosphate mainly via induction of the metabolism through the secretion and oxidation of gluconic acid. The proposed mechanism of phosphate-solubilization in *Pantoea eucalypti,* under our experimental conditions, is summarized in Fig. 6.

As a final point, considering the comments made above, current study is in progress to evaluate under different field conditions the performance of this native strain and its relationship with native (nitrogen-fixing and P-solubilizing) soil micro-organisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Influence of bacterial inoculation on *Lotus tenuis* growth. Seedlings were inoculated in semisolid Evans medium, under phosphate deficiency, using a group of isolates with high *in vitro* phosphate-solubilization activity identified as *Pantoea eucalypti* (M91), *Pseudomonas fluorescens* (M25) and *Erwinia rhapontici* (M52). Data was expressed as relative shoot dry weight (%) (shoot dry weight of inoculated plants x 100/shoot dry weight of non-inoculated plants). Different letters indicate significant difference according to Tukey's method at $P = 0{\cdot}002$ (*post hoc* testing). Data represent mean ± SD ($n = 5$, i.e. five replicate flasks per treatment containing four plants each).

Figure S2 Plant growth promoting activity of isolate M91. Seedlings were transferred to tubes containing 5 ml of semisolid modified Evans medium and inoculated with a bacterial water suspension.

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