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Plant-associated fluorescent *Pseudomonas* from red lateritic soil: Beneficial characteristics and their impact on lettuce growth

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Fluorescent *Pseudomonas* are ubiquitous soil bacteria that usually establish mutualistic associations with plants, promoting their growth and health by several mechanisms. This makes them interesting candidates for the development of crop bioinoculants. In this work, we isolated phosphate-solubilizing fluorescent *Pseudomonas* from the rhizosphere and inner tissues of different plant species growing in red soil from Misiones, Argentina. Seven isolates displaying strong phosphate solubilization were selected for further studies. Molecular identification by *rpoD* genotyping indicated that they belong to different species within the *P. fluorescens* and *P. putida* phylogenetic groups. Screening for *in vitro* traits such as phosphate solubilization, growth regulators synthesis or degradation, motility and antagonism against phytopathogens or other bacteria, revealed a unique profile of characteristics for each strain. Their plant growth-promoting potential was assayed using lettuce as a model for inoculation under controlled and greenhouse conditions. Five of the strains increased the growth of lettuce plants. Overall, the strongest lettuce growth promoter under both conditions was strain ZME4, isolated from inner tissues of maize. No clear association between lettuce growth promotion and *in vitro* beneficial traits was detected. In conclusion, several phosphate solubilizing pseudomonads from red soil were isolated that display a rich array of plant growth promotion traits, thus showing a potential for the development of new inoculants.

Key Words: PGPR; phosphate solubilization; plant growth promotion; *Pseudomonas*

Introduction

Beneficial bacteria that interact with plants, termed plant growth-promoting rhizobacteria (PGPR), can be found in the soil, rhizosphere, or interior of plant tissues, establishing mutualistic associations as endophytes (Rout, 2014). Fluorescent *Pseudomonas* have been classically isolated from the soil and rhizosphere and subjected to extensive characterization as biocontrol agents and PGPR (Lucy et al., 2004). Although, in comparison, *Pseudomonas* endophytes have been less studied, some microbial diversity surveys have revealed that they are a fundamental part of the microbiome of plants (Hallmann and Berg, 2006). Thus, it should not be surprising if endophytic pseudomonads are able to establish a closer association with their hosts while promoting plant growth.

The efficient colonization of plant roots by *Pseudomonas* is a prerequisite for plant growth promotion (PGP). This, in turn, depends on multiple determinants such as motility (Martínez-Granero et al., 2006), biofilm formation (Bogino et al., 2013), nutrient scavenging (Ghirardi et al., 2012), and communication factors (Wei and Zhang, 2006). Once the association has been established, plants might benefit from endophytes through different PGP mechanisms. Manipulation of the levels of plant growth regulators is thought to be an important mechanism underlying PGP by fluorescent *Pseudomonas*. For example, the synthesis of the auxin indole-acetic acid (IAA) or the catalysis of ACC, an ethylene precursor, by PGPR positively

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Table 1. Information about the *Pseudomonas* strains isolated in this work.

Strain	Habitat	Plant source	Phylogenic group	Closest species*	*rpoD*
LSR1	Rhizospheric	Lettuce (cv. Great Lakes)	*P. putida*	*P. hunanensis*	KT284712
MME1	Endophytic	Tomato (cv. Moneymaker)	*P. fluorescens fluorescens*	*P. synxantha*	KT284709
MME3	Endophytic	Tomato (cv. Moneymaker)	*P. fluorescens koreensis*	*P. koreensis*	KT284710
MTR4	Rhizospheric	Tomato (cv. Micro-Tom)	*P. putida*	*P. putida*	KT284713
TAE4	Endophytic	Wheat (cv. Tauro)	*P. fluorescens fluorescens*	*P. extremorientalis*	KT284711
TAR5	Rhizospheric	Wheat (cv. Tauro)	*P. fluorescens jessenii*	*P. jessenii*	KT284714
ZME4	Endophytic	Maize (cv. Pannar RR)	*P. fluorescens fluorescens*	*P. rhodesiae*	KT284708

*According to BLAST comparison.

influences root growth and impacts on the final yield of plants (Belimov et al., 2009; Egamberdieva, 2012). In addition, some *Pseudomonas* are capable of producing other plant growth regulators, such as cytokinins (García de Salamone et al., 2001), giberellic acid (Karakoç and Aksöz, 2006), absicic acid (Sgroy et al., 2009), but their role in plant growth promotion is not yet clear.

Another characteristic that has brought these bacteria to the attention of researchers is their recurrent capacity to solubilize fixed forms of phosphates through the release of organic acids (Rodríguez and Fraga, 1999). Several studies have shown that inoculation with phosphate-solubilizing strains of *Pseudomonas* can improve plant phosphorous nutrition under different fertilization conditions (Fankem et al., 2008; Yu et al., 2011). In addition, fluorescent *Pseudomonas* usually suppress plant diseases by antagonizing phytopathogens as an indirect plant-growth promoting trait.

Numerous factors have been identified as being responsible for plant protection, such as the release of secondary metabolites, extracellular proteins, lytic enzymes and scavenging molecules like siderophores. Most of these factor have a direct effect on phytopathogens, but some of them act indirectly by eliciting a generic defence mechanism in the plants known as the "induced systemic resistance" or ISR (Compant et al., 2005). All this evidence underlines the potential of crop inoculation with *Pseudomonas* as a means to increase productivity while reducing chemical fertilization, in the context of a healthier and sustainable agriculture.

The objective of this work was to isolate plant-associated phosphate-solubilizing *Pseudomonas* from lateritic soil of high iron content and to examine their potential as PGPR. To accomplish this, fluorescent pseudomonads were recovered from the rhizosphere and inner tissues of different plant species growing on red soil. The characterization of their PGP traits *in vitro* and *in vivo*, using lettuce as a model for inoculation, is presented.

Material and Methods

Isolation and growth conditions. *Pseudomonas* sp. were isolated from the rhizosphere or whole body of 10-day plantlets grown in red lateritic soil from Misiones province (Argentina). Seeds of wheat (*Triticum aestivum* L. cv. Tauro), maize (*Zea mays* L. cv. Pannar RR), tomato (*Solanum lycopersicum* L. cv. Moneymaker and cv. Micro-Tom) and lettuce (*Lactuca sativa* L. cv. Great Lakes) were disinfected superficially by treating with 70% ethanol for 1 min and then with 1% NaOCl for 5 min, rinsed 5 times with sterile distilled water (SDW) and sown in pots filled with soil. After approximately 10 days of growth, some plants were harvested, cleaned superficially to remove the adhered soil from the roots and incubated in a sterile saline solution (SS) for 1 h under soft shaking to obtain rhizosphere extracts. The remaining plants were thoroughly disinfected with 70% ethanol for 1 min and 1% NaOCl for 20 min with vigorous shaking, and then homogenized in 2 mL of SS to obtain tissue extracts. Both rhizosphere and tissue extracts were diluted serially and 100 μl of each dilution were plated on *Pseudomonas* selective medium S1 (Gould et al., 1985). After 48 h of incubation at 30°C, the plates were examined for colonies of distinct phenotypes, which were sub-cultured in S1 medium for further selection and purification.

Pseudomonas isolates were routinely cultured on a King B (King et al., 1954) derivative medium termed ER medium (containing per liter: 10 g tryptone, 10 mL glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$; pH = 7). Unless specified, all spot-inoculations on plates were carried out by seeding drop-wise with 3 μL of fresh cultures of the bacteria in a stationary phase. Viable cells in starter cultures and inoculants were quantified by the drop method (Miles et al., 1938) in ER-agar medium.

Molecular identification. The selected isolates were genotyped by sequencing a fragment of the *rpoD* gene as detailed by Mulet et al. (2009). PCR-amplified fragments were separated by agarose gel electrophoresis, purified with QIAEX II gel extraction kit (Qiagen) and automatically sequenced by the Genomics Department of the Biotechnology Institute, INTA-Castelar, Argentina. The resulting sequences were deposited in the Genbank database (Table 1) and compared to other sequences available in the database with the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/). Further phylogenetic analyses were carried out with the MEGA 5.2 software (Tamura et al., 2011) using the Maximum Likelihood method, Tamura-Nei substitution model and a bootstrap phylogeny test of 1000 iterations. Sequences of other *Pseudomonas* (Mulet et al., 2010, 2013) were retrieved from Genbank.

Phosphate solubilization. Qualitative phosphate solubilization assays were performed by spot-inoculating NBRIP medium plates (Nautiyal, 1999), amended with 3 $g \cdot L^{-1}$ hydroxyapatite (Biorad) as the source of insoluble phosphate. Solubilization was estimated after 4 days of incubation at 30°C by measuring the diameter of the transparent halo around colonies.

Quantitative determination of phosphate solubilitation was carried out in NB-RIP liquid medium, amended with 3 g·L^{-1} of hydroxyapatite, aluminium phosphate (Sigma-Aldrich) or patagonic phosphate rock (Forminar SRL, Argentina). The phosphate rock powder was previously washed 3 times with SDW and dried at 40°C to remove residual soluble phosphates. Test tubes containing 3 mL of NB-RIP medium were inoculated with 0.1 mL of *Pseudomonas* starter cultures containing approximately 1·10^8 CFU·mL^{-1}, and incubated at 30°C with shaking at 200 rpm for 24 h. Three independent replicates were used for each treatment, and non-inoculated tubes were included as the control treatment. Afterwards, samples were centrifuged at 12,000 rpm for 5 min and 1 mL of the supernatant was stored at –20°C until use. When required, the supernatants were diluted with distilled water. Soluble phosphate was quantified colorimetrically by the ascorbic acid method (Murphy and Riley, 1962) in a final reaction volume of 270 μL in microplates and a measurement at 880 nm with a Multiskan Spectral spectrophotometer (Thermo Scientific).

Determinations of plant growth regulators. Production of IAA was estimated by the Salkowski method (Pilet and Chollet, 1970). Test tubes containing 4 mL of King B medium were inoculated with 0.1 mL of starter cultures and incubated at 30°C with shaking at 200 rpm. After 72 h, 1 mL of each supernatant was mixed with 1 mL of Salkowski reagent (74 mM $FeCl_3$ in 7.9 M H_2SO_4) and incubated at 30°C in the dark for 30 min. Absorbance was measured at 530 nm and IAA concentration was determined by extrapolating to a standard curve.

ACC deaminase activity (ACCd) was measured following the method described by Penrose and Glick (2003) adapted to microplates. *Pseudomonas brassicacearum* DBK11^T (Long et al., 2008) was used as a positive control.

For both assays, uninoculated tubes served as the blank treatment and three independent replicates for each treatment were carried out.

Fungal and bacterial antagonism. Antagonism against plant pathogenic fungi was evaluated *in vitro* by quantifying the inhibition of mycelial growth by the *Pseudomonas* strains, in Sabouraud medium (Oxoid) plates. Agar plugs of 8 mm diameter, excised from full-grown plates of *Rhizoctonia solani* AG-3, *Fusarium solani* var. *eumartii* or a field isolate of *Sclerotinia sclerotiorum* (Lib.) de Bary, were placed on the centre of a fresh plate and the bacterial strains were spotted at a fixed distance from the fungi. *R. solani* and *F. solani* were grown at 30°C while *S. sclerotiorum* was grown at 20°C. After 48 h, antagonism was revealed as a growth inhibition halo around bacterial colonies and estimated by comparing mycelial growth in the direction to the *Pseudomonas* with the control growth in the opposite direction. Experiments were performed in six independent replicates.

To determine the antagonism against other bacterial species, solid media in Petri dishes was inoculated by spreading 0.5 mL of reference bacteria saturated cultures to grow as a loan. After drying, the different *Pseudomonas* strains were spotted and the plates were incubated at 30°C for 48–96 h. Growth inhibition was diagnosed by measuring the exclusion halo around *Pseudomonas* colonies. *Agrobacterium tumefaciens* B6-S3 (Vervliet et al., 1975), *P. syringae* pv. *tabaci* 6605 (Taguchi et al., 2001) and *Azospirillum brasilense* Az39 (Rodriguez Caceres et al., 2008) assays were carried out on LB media (Bertani, 1951), and experiments with *Bradyrhizobium japonicum* E109 (Perticari et al., 1996) were carried out on M79 medium (Fred and Waksman, 1928).

Motility assay. The motility of the isolated strains was examined in a plate swimming assay. Bacterial suspensions were spot-inoculated with a toothpick in the center of a Petri dish containing 0.3% agar KingB medium. After 16 h of incubation at 30°C, motility was estimated by measuring the radii of swimming halos.

Other biocontrol traits. Production of siderophores was assayed by the CAS agar assay (Schwyn and Neilands, 1987) employing ER medium nutrients instead of the original ones. The plates were spot-inoculated with the *Pseudomonas* strains and incubated at 30°C for 48 h until an orange halo developed around colonies. The production of siderophores was estimated by a relative index (halo diameter·colony diameter^{-1}).

Hydrogen cyanide (HCN) production was determined as described by Egan et al. (1998). Bacterial cultures were spotted on ER-agar medium supplemented with 4 g·L^{-1} glycine. A filter paper impregnated with a solution of 0.5% picric acid in 2% Na_2CO_3 was placed in the lid of the plates. After 48 h of incubation at 30°C, the filter paper color change from yellow to orange-brown indicated HCN production. Uninoculated plates were used as a negative control.

Lettuce growth promotion experiments. To obtain bacterial inocula, 5 mL of ER medium in glass tubes were seeded with 50 μL of starter cultures of the *Pseudomonas* strains and grown at 30°C and 200 rpm shaking for 18 h. A viable cell count showed that these inocula contained between 1·10^9 and 5·10^9 CFU·mL^{-1}.

For the growth promotion trial in a controlled environment, seeds of lettuce (cv. Great Lakes) were disinfected as explained before and sown in 180 cm^3 pots filled with vermiculite previously sterilized. At emergence, 1 mL of inoculum was applied at the base of the stem of each plantlet. Plantlets treated with SS were used as the control group. Each treatment consisted of 7 independent replicas that were arranged in a completely randomized block design. Plants were incubated for 7 more days in a growth chamber under controlled light and temperature according to the ISTA Standard (International Seed Testing Association, 2015) and watered with rainwater as needed. Afterwards, plants were carefully removed, washed and examined for growth promotion by measuring the length of the root system, the height and fresh weight of the aerial portion and the root and aerial portion dry weight after drying at 60°C until constant weight was noted.

Lettuce growth promotion was also assayed in a greenhouse with natural light during the autum-winter season. Lettuce seeds (cv. Gallega de invierno) were superficially disinfected and germinated in a seedbed of sterile vermiculite. At emergence, seedlings were transplanted to

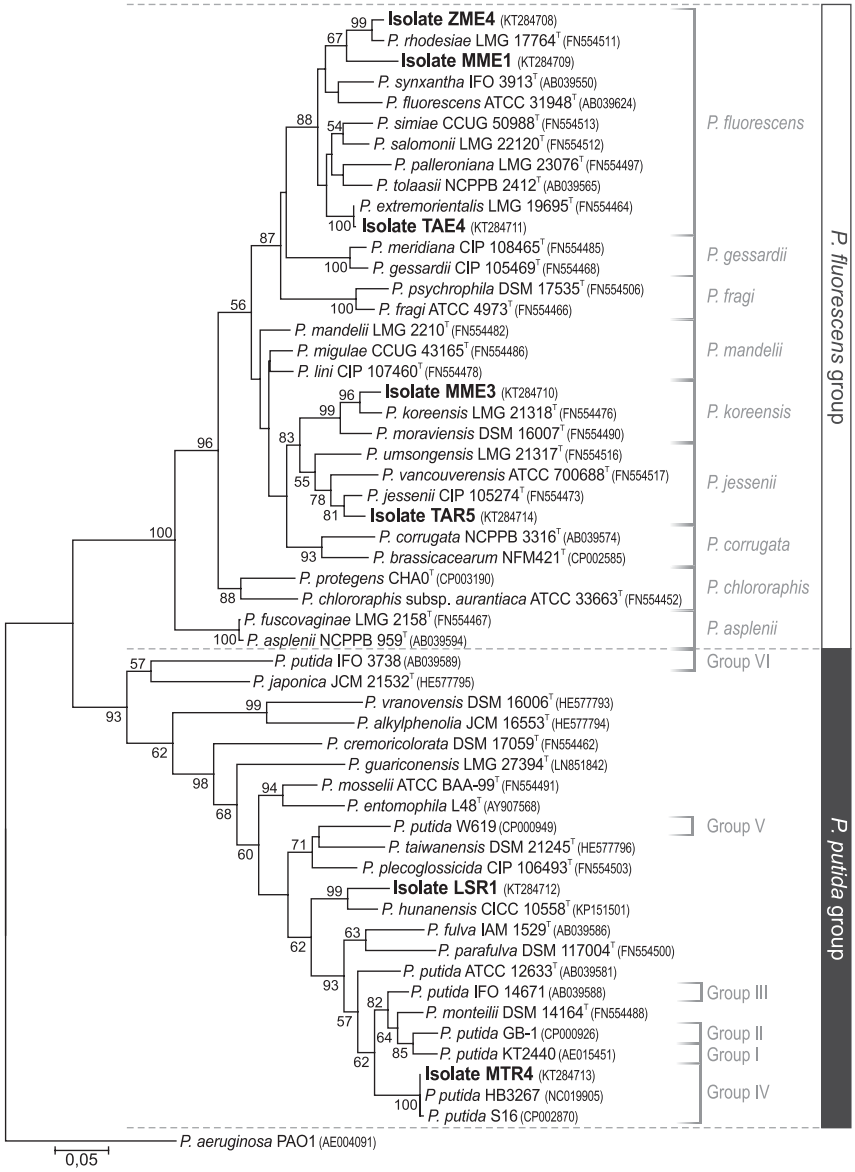

Fig. 1. Phylogenetic analysis of the *Pseudomonas* isolates.

Phylogenetic tree inferred from the *rpoD* gene sequences of the isolates (highlighted in bold) and other representative *Pseudomonas*. Sequences were subjected to multiple-alignment with ClustalW and tree inference by the Maximum Likelihood method, under the Tamura-Nei model. Numbers at the nodes are percentage bootstrap values ≥50% based on 1000 resampled datasets. *P. fluorescens* and *P. putida* phylogenetic groups and their subgroups are indicated as described by Mulet et al. (2010, 2013). *P. aeruginosa* PAO1 was used as an out-group. Genbank accession codes for the sequences employed in the analysis are detailed in brackets next to each name.

180 mL pots, containing a 1:1 mixture of vermiculite and rich soil, and inoculated at the base of the stem with 1 mL of bacterial suspension containing approximately 10^9 CFU·mL^{-1}. Plants inoculated with SS were used as the control treatment. Plantlets were raised for 10 more days under controlled conditions and then transplanted to 3 L pots, re-inoculated with 1 mL of inoculum and transferred to the greenhouse. Treatment was independently replicated 15 times and arranged in a completely randomized way. At harvest time, plants were carefully removed from the pots, washed and examined for growth promotion as explained above.

Statistical analysis. All the data was analysed using GraphPad Prism 5 (GraphPad Software Inc., California, USA) or Infostat (http://www.infostat.com.ar) using one-way ANOVA plus Tukey's post-test, or by the Kluskal-Wallis non-parametric test plus Dunn's post-test when the data did not fit the required parameters of ANOVA. The *t*-test was also employed for paired analysis of specific cases. Differences were considered to be significant at $p < 0.05$.

Results

Isolation and molecular identification of fluorescent Pseudomonas

We established a collection of endophytic and rhizospheric *Pseudomonas* sp. isolates associated with different plant species grown on red lateritic soil. From

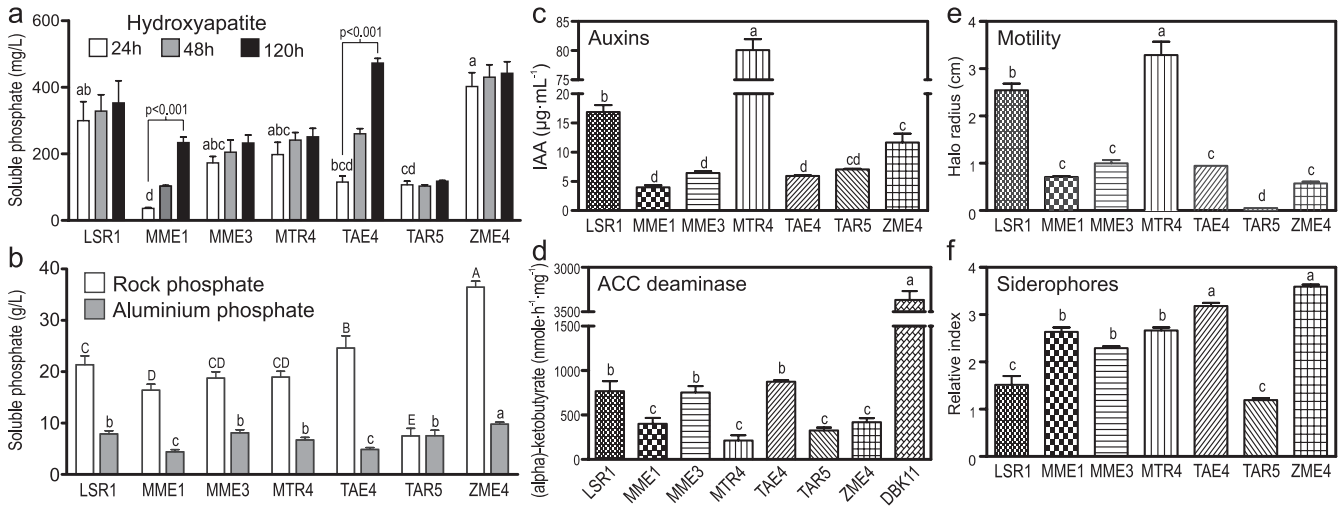

Fig. 2. Characterization of *in vitro* PGP traits.

(a), (b) Soluble phosphates in the supernatant of the *Pseudomonas* strains released from hydroxyapatite after 24, 48 and 120 h (a), and from rock phosphate or aluminium phosphate after 24 h (b). (c) *In vitro* production of IAA after 72 h of growth in King B medium, as estimated by a colorimetric method. (d) ACC deaminase activity of toluene-permeabilized bacteria after a 24 h induction with ACC. *P. brassicacearum* DBK11 was used as a positive control. (e) Release of siderophores by the *Pseudomonas* strains in a CAS agar assay, estimated as orange halos around colonies. (f) *In vitro* motility of the isolated strains analysed by a swimming assay on 0.3% agar King B medium. Plotted data are mean values of three independent replicates per treatment and error bars indicate standard deviation. In all the cases, data was grouped according to Tukey's test performed after one-way ANOVA. Paired comparisons were done by the *t*-test (a).

approximately 70 isolates that were recovered from both habitats, 7 phenotypically distinct isolates were selected for further characterization based on a qualitative screening for hydroxyapatite phosphate solubilization in NB-RIP solid medium (data not shown). These isolates were named LSR1, MME1, MME3, MTR4, TAE4, TAR5 and ZME4.

Genotyping by sequencing a fragment of the *rpoD* gene revealed that the isolates are distinct strains of the *P. fluorescens* and *P. putida* groups (Fig. 1). The *P. fluorescens* strains distributed within the *fluorescens* (MME1, TAE4 and ZME4), *koreensis* (MME3) and *jessenii* (TAR5) sub-groups in the phylogenetic tree (Fig. 1). When compared with other *Pseudomonas* species using BLAST, the closest matches for MME1, MME3, TAE4, TAR5 and ZME4 were *P. synxantha* IFO 3913^T (92% identity), *P. koreensis* LMG 21318^T (96% identity), *P. extremorientalis* LMG 19695^T (99% identity), *P. jessenii* CIP 10574^T (97% identity) and *P. rhodesiae* LMG 17764^T (98% identity), respectively. Regarding strains within the *putida* group, the closest matches for LSR1 and MTR4 were *P. hunanensis* CICC 10558^T (94% identity) and *P. putida* HB3267 (99% identity), respectively. Information about the isolated *Pseudomonas* strains, origin, proposed phylogeny and Genbank accession codes for *rpoD* sequences is detailed in Table 1.

Screening for plant growth promotion traits

Phosphate solubilization by the isolated strains was analysed in liquid media using three different insoluble compounds: hydroxyapatite, aluminium phosphate and rock phosphate. A 5-day time curve of hydroxyapatite solubilization indicated that ZME4 is the strongest solubilizer at day 1, with a mean production of 402.5 ± 72.04 mg·L^{-1} soluble phosphate (Fig. 2a). Strains LSR1, MME3, MTR4, TAR5 and ZME4 were rapid solubilizers but did not maintain this activity for long, as evidenced by the negligible increase in soluble phosphate after 24 h. On the other hand, the solubilization activity of MME1 and TAE4 was relatively constant, resulting in a significant increase of soluble phosphate ($p < 0.001$ in both cases) after 3 days of growth (Fig. 2a). The differential behaviour of these two groups may be explained by differences in bacterial survival since values of CFU·mL^{-1} throughout the assay remained stable for MME1 and TAE4 but declined for the rest of the strains (data not shown). Experiments using rock phosphate or aluminium phosphate as the phosphate insoluble source were carried out only for 24 h. In these assays, ZME4 was again the most active solubilizer by producing significantly higher amounts of soluble phosphate than other strains (Fig. 2b). Overall, ZME4 showed the strongest phosphate solubilization activity at 24 h in all the tested conditions.

An assay for *in vitro* auxin production in liquid media indicated that the seven *Pseudomonas* strains were capable of synthesizing auxins after 3 days of growth in King B medium. MTR4 showed the highest mean accumulation of IAA in the supernatant (80.06 ± 3.25 µg·mL^{-1}), followed by LSR1 (16.87 ± 2.08 µg·mL^{-1}) and ZME4 (11.65 ± 2.61 µg·mL^{-1}) (Fig. 2c).

ACCd was detected in all the strains but at much lower levels than *P. brassicacearum* DBK11^T used as the positive control. Two groups with significantly different ACCd were observed within the isolated strains (Fig. 2d).

The motility of the *Pseudomonas* was examined in swimming assays with semisolid King B medium. Strain TAR5 showed no motility, while the rest produced clearly visible halos due to swimming from the inoculation point. Strains MTR4 and LSR1 produced the largest swimming halos (Fig. 2e).

Analysis of biocontrol properties

Siderophores and hydrogen cyanide (HCN) production

Table 2. HCN production and antagonism to phytopathogens and other rhizobacteria.

	LSR1	MME1	MME3	MTR4	TAE4	TAR5	ZME4	Pf-5
HCN production	–	–	+	–	–	+/–	–	+
Antagonism								
Rhizoctonia solani	++	–	–	+	–	+	++	+
Fusarium solani	++	–	–	+	–	+	++	+
Sclerotinia scelrotiorum	–	+	+	–	+	–	–	+
Pseudomonas syringae	+	+	–	+	+	–	–	+
Agrobacterium tumefaciens	–	–	–	–	–	–	–	+
Azospirillum brasilense	–	–	–	–	+	–	+	+
Bradyrhizobium japonicum	–	–	–	–	+	–	–	+

+ positive; – negative; +/– inconsistent results; ++ strongly positive.

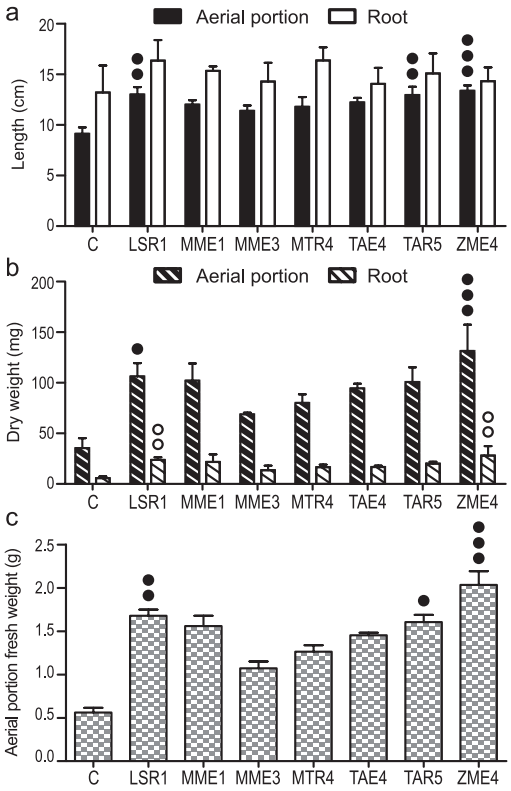

Fig. 3. Lettuce growth promotion in a controlled environment.

Effect of inoculation with the *Pseudomonas* strains on the growth of lettuce plants under controlled environmental conditions. After 10 days in the growth chamber, plantlets were analysed for differences in growth by measuring the length (a) and dry weight (b) of both root system and aerial portion, and the fresh weight of the aerial organs (c). Error bars indicate standard deviation. The black dots indicate the level of significance according to the Kluskal-Wallis non-parametric test followed by Dunn's test.

was assayed. All the strains secreted siderophores in the CAS agar assay. Strains TAE4 and ZME4 produced the largest orange halos and strains LSR1 and TAR5 were weak producers of these compounds (Fig. 2f). On the other hand, MME3 was the only strain that consistently produced HCN; the production of this volatile by TAR5 was erratic (Table 2).

Antagonism assays against fungal phytopathogens indicated that *R. solani* and *F. solani* growth was inhibited by the presence of strains LSR1, MTR4, TAR5 and ZME4 but was poorly affected by strains MME1, MME3 and TAE4 (Table 2). Inversely, *S. sclerotiorum* development was impaired only by strains MME1, MME3 and TAE4.

When analysing the effect of the *Pseudomonas* strains on the growth of several bacteria species *in vitro*, we observed that strains LSR1, MME1, MTR4 and TAE4 inhibited *P. syringae* growth, while *A. tumefaciens* growth was unaffected by the presence of any of the isolated strains in the media. The growth of the plant-beneficial bacteria *A. brasilense* was impaired by TAE4 and ZME4, whereas only TAE4 interfered with *B. japonicum* growth (Table 2). As expected, *P. protegens* Pf-5 used as a positive control was an effective biocontrol agent in all of the cases tested (Table 2).

Plant growth promotion by the fluorescent Pseudomonas

Experiments were carried out to analyse the effect of the *Pseudomonas* strains on the growth of lettuce plants under both environmentally controlled and greenhouse conditions.

Under environmentally controlled conditions, growth promotion was most notably observed after inoculation with the lettuce rhizospheric strain LSR1 and the maize endophytic strain ZME4. These strains significantly increased the length, dry weight and fresh weight of the aerial portion, as well as the root dry weight (Fig. 3). The wheat rhizospheric strain TAR5 also promoted lettuce growth by inducing a significant increase in the length and fresh weight of the aerial portion (Figs. 3a and 3c).

In the greenhouse trial, the aerial portion of plants showed a higher fresh weight when inoculated with MME3 (Fig. 4a), and an increased dry weight when treated with MME1 or ZME4 (Fig. 4b). Strain LSR1 also showed a tendency to increase the fresh and dry weight of the aerial organs, but the differences were not statistically significant (Figs. 4a and 4b). Similarly, no statistically significant effect was observed in the root growth parameters when the *Pseudomonas* treatments were compared to the control, although strains MME3 and ZME4 showed a tendency to increase root length and dry weight, respectively (Figs. 4c and 4d).

Discussion

Plant-associated *Pseudomonas* that inhabit the rhizosphere, rhizoplane, or inner tissues of plants as endophytes, have been shown to positively affect plant

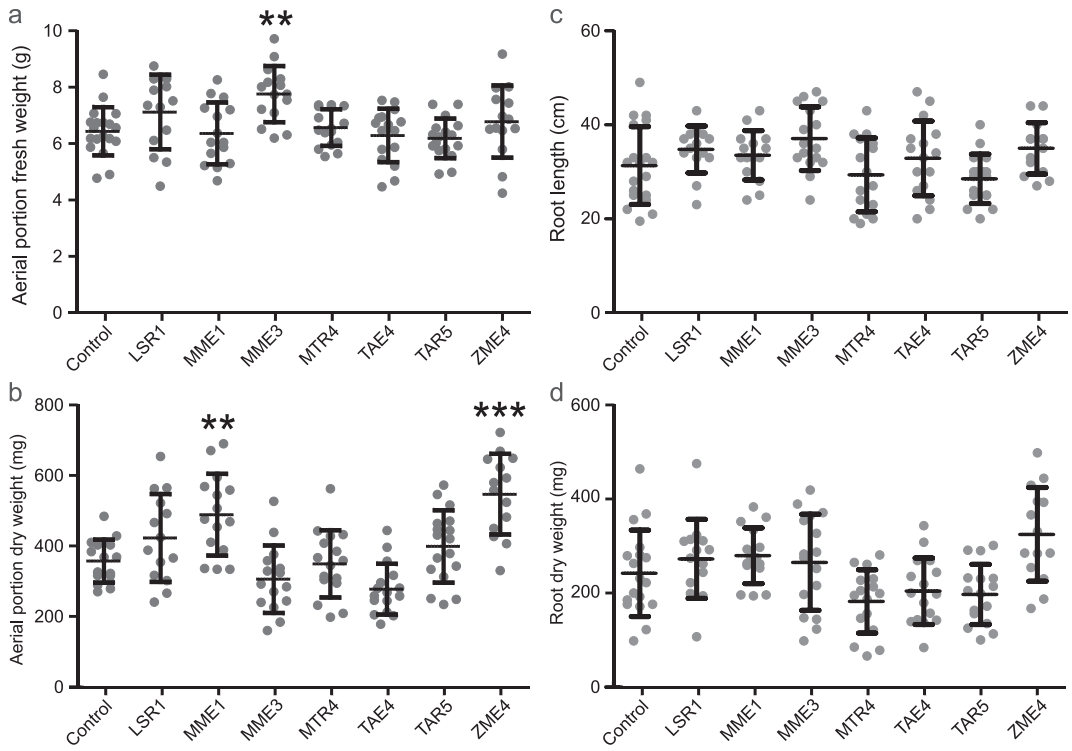

Fig. 4. Lettuce growth promotion in greenhouse.

Effect of inoculation with the fluorescent *Pseudomonas* on the growth of lettuce under greenhouse conditions. Plants were inoculated with two independent applications (at emergence and at transplantation) and grown under natural light and temperature in the greenhouse. At harvest time, their growth was analysed by measuring fresh (a) and dry weight (b) of the aerial organs, and length (c) and dry weight (d) of the root system. Bars in each scatter plot indicate mean and standard deviation. Black asterisks indicate the level of significance according to Tukey's test performed after one-way ANOVA.

growth, health and nutrition. Diverse mechanisms mediate this effect, including the modulation of plant growth regulators levels, transformation of insoluble or fixed nutrients within the soil into bio-available forms and protection against phytopathogens (Choudhary et al., 2009; Richardson et al., 2009). In this work, we isolated several *Pseudomonas* sp. strains from the rhizosphere and inner tissues of four economically important crop species grown in red lateritic soil. Considering that plants were strongly disinfected before extraction, which led to a poor recovery of colonies, only bacteria located within deep tissues might have survived. Consequently, the isolates recovered from whole plants are here referred to as endophytes, although the true endophytic habit of the strains would need confirmation by microscopy.

Seven of the recovered isolates showed strong solubilization of hydroxyapatite in a qualitative screening assay and, consequently, were selected for further study. Molecular genotyping of these isolates was carried out by partial sequencing of the *rpoD* gene due to its convenient characteristics as a phylogenetic marker in *Pseudomonas* (Mulet et al., 2009). Sequence comparison and phylogenetic analysis indicated that strains MME3, TAE4, TAR5 and ZME4 should be considered to be members of the *P. koreensis*, *P. extremorientalis*, *P. jessenii* and *P. rhodesiae* species within the *P. fluorescens* group. Although results for MME1 differed between BLAST and phylogenetic analysis, its low nucleotide identity to described type strains (92%) indicate that MME1 might belong to a yet undescribed species. On the other hand, LSR1 and MTR4 were detected as members of the *P. putida* group. Sequence comparison suggest that MTR4 belongs to a separate sub-group termed species IV by Mulet et al. (2013), and that LSR1 might be a member of a yet undescribed species close to *P. hunanensis* (94% nucleotide identity). Although the *rpoD* gene allowed an accurate identification of most *Pseudomonas* strains, a multilocus sequence analysis including other genes such as 16S rRNA and *gyrB* would support their classification, especially in the case of MME1.

Characterization of the isolated *Pseudomonas* strains showed that the each strain displays a distinct profile of PGP traits. Strain ZME4 was a rapid and efficient phosphate solubilizer of three different substrates: hydroxyapatite, aluminium phosphate and rock phosphate. Various evidence shows that inoculation with phosphate-solubilizing *Pseudomonas* can lead to higher phosphorous levels in treated plants (Fankem et al., 2008; Yu et al., 2011). In the case of ZME4, the persistence of phosphate solubilization under natural conditions and its impact on plant nutrition remains to be tested in future experiments.

The synthesis and secretion of auxins is one of the most studied mechanisms underlying PGP by rhizobacteria (Spaepen et al., 2007). Strain MTR4 produced, comparatively, the highest levels of auxins *in vitro* but did not significantly increase the growth of lettuce plants in the two assayed conditions. These results indicate that *in vitro* production of auxins does not reflect the behaviour of the

strains *in planta*, or that these compounds do not influence plant growth substantially, at least under our experimental conditions. Similarly, there was no obvious influence of ACCd on inoculated lettuce plants. The swimming motility of the *Pseudomonas* strains was also assayed due to its importance in the competitive colonization of plant roots. It has been shown that mutant strains defective in flagella production, and consequently reduced in swimming motility and chemotactic responses, show a reduced fitness in plant growth-promotion (Turnbull et al., 2001). We observed that two rhizospheric *P. putida* strains, LSR1 and MTR4, were highly mobile in the swimming assay. This is expected since the rhizosphere is an ecological niche that hosts a large and rich community of microbes and only highly competitive strains will be successful in surviving these conditions (Prashar et al., 2014). On the contrary, the third rhizospheric strain TAR5 was mostly immobile, but this result might be explained by its extremely mucous phenotype when grown in King B medium (data not shown). Overall, the results showed that motility of the isolated *Pseudomonas* is not associated with lettuce growth promotion under our experimental conditions.

Production of siderophores, iron-chelating molecules that hijack the available iron in the media, and HCN, a broad-spectrum antimicrobial compound, are two well-documented biocontrol traits of PGPR (Mossialos and Amoutzias, 2007; Ramette et al., 2003). Our results suggest that siderophore production might be associated with competition with the plant-beneficial diazotroph *A. brasilense* and *B. japonicum*. In agreement, after multiple correspondence analyses, Ghirardi et al. (2012) concluded that one of the most important characteristics of fluorescent *Pseudomonas* in rhizosphere competence is siderophore-mediated iron acquisition. This information is relevant for the formulation of *Pseudomonas* co-inoculants with *rhizobia* or *Azospirillum* sp. where the compatibility of strains is critical (Trabelsi and Mhamdi, 2013). On the other hand, growth inhibition of *P. syringae* by some of the strains might be related to other factors important for intra-genera competition since none of the *Pseudomonas* was effective in controlling the growth of a tumorigenic *A. tumefaciens*. Notably, HCN synthesis could not be associated with the biocontrol of bacterial growth *in vitro*.

Biocontrol of phytopathogenic fungus was neither associated with production of siderophores nor HCN. Nevertheless, some of the *Pseudomonas* strains were as effective as *P. protegens* Pf-5, a well-studied biocontrol agent (Paulsen et al., 2005), in controlling the growth of the phytopathogens. Biocontrol profiles obtained against *R. solani* and *F. solani* were similar, and inverse to that obtained for *S. scelotiorum*. This implies the existence of different and independent mechanisms underlying fungal biocontrol by these strains, possibly involving secondary metabolites and/or lytic enzymes as has been described for other *Pseudomonas* (Compant et al., 2005). Interestingly, recent evidence has pointed to cyclic-lipopeptides as key factors in the biological control of *R. solani* AG-3 by *P. fluorescens* (Michelsen et al., 2015).

To characterize the plant growth-promotion capacity of the *Pseudomonas* strains, lettuce was selected as an experimental model. Growth promotion profiles under a controlled environment were different than those in the greenhouse, in which inoculation effects were less significant. The use of a sterile substrate might account for the greater effects observed in the growth chamber trial. Plants in the greenhouse experiment were raised using a rich soil containing native populations of microorganisms that might possibly interact with the inoculated bacteria and plants, thus modifying the outcome of the experiments. Indeed, under natural circumstances, only the most competitive and adapted bacterial strains will effectively colonize the roots and establish a successful plant-bacteria interaction (Weller and Thomashow, 2007). Our results support the decisive role of natural microbial communities within the soil in shaping the effectiveness of inoculants.

The maize endophytic strain ZME4 was the most consistent growth promoter of lettuce plants in both tested conditions, suggesting that its mechanisms of root colonization and growth promotion are not specific to a plant genotype. This mode of action has been also demonstrated for other PGPR strains. For example, Zinniel et al. (2002) showed that endophytes isolated from corn and sorghum were able to colonize several non-related plant species. All these evidences support the existence of generic mechanisms of plant growth promotion by PGPR.

The present work settles an important precedent on the characterization of seven fluorescent *Pseudomonas* strains from red soil that were found associated with plants. Our exploratory approach revealed that the isolated strains are phylogenetically distinct and display a unique profile of beneficial characteristics, as well as a good potential for plant inoculation. Molecular and biochemical approaches will be necessary to understand the basis of the differential characteristics of the strains. From an agronomic point of view, our results may lead the way to a more profound exploration of these *Pseudomonas* concerning *in vivo* biocontrol capacity, impact on phosphorous uptake, and plant growth-promotion in the field.

Conclusions

New strains of *Pseudomonas* that belong to the *fluorescens* and *putida* phylogenetic groups were recovered from the rhizosphere and inner tissues of different plant species. These strains displayed diverse profiles of *in vitro* PGP characteristics commonly exhibited by plant growth-promoting rhizobacteria. Some of them were also able to promote the growth of lettuce plants under a controlled environment and in a greenhouse. These effects differed between the different experimental conditions and were not associated with any of the bacterial *in vitro* PGP characteristics. Other factors, single or in combination, might be modulating bacterial competitiveness and/or plant-bacteria interaction. The isolated strains should be tested under productive conditions to assay their potential as crop inoculants.

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