

# Plant growth-promoting rhizobacteria able to improve NPK availability: selection, identification and effects on tomato growth

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## ABSTRACT

Nitrogen, P and K are essential macronutrients that are not readily available to plants. Rhizobacteria are able to convert these unavailable forms for subsequent uptake by the plant, diverse species have been characterized as N<sub>2</sub> fixers, P solubilizers and capable to solubilize mineral K from unavailable forms. The objective of this study was to select rhizobacteria capable of improving NPK availability and promoting tomato (*Solanum lycopersicum* L.) growth. Fifteen strains were studied. Four strains were selected for their capacity to fix N<sub>2</sub>, three for their ability to solubilize P, and six for their capacity to solubilize biotite and K-feldspar, isolated from tomato, lentil, chili pepper, faba bean and lettuce crops in Andisol and Alfisol soils. Through 16S rRNA sequencing, selected strains were identified as *Pseudomonas gessardi*, *P. koreensis*, *P. brassicacearum*, *P. marginalis*, *Acinetobacter calcoaceticus* and *Rahnella aquatica*. Phosphorus solubilizing strains did not show a positive effect on plant growth or an increase in available soil P. The N<sub>2</sub> fixing bacteria Tmt-16 strain increased root growth in 23.57%; maintained the highest N content in plant tissue, 2.60%, higher amount of N available in the soil, 2.95 mg kg<sup>-1</sup>, and a higher content of N-NH<sub>4</sub><sup>+</sup> 1.95 mg kg<sup>-1</sup>. The K solubilizing strains Ls-C21, Ltj-62 and LsC-58 reached 17.0 to 19.0 mg kg<sup>-1</sup> available K and 0.04 to 0.05 mg kg<sup>-1</sup> exchangeable K ( $p \leq 0.05$ ). These four endemic rhizobacteria can be potentially used as biofertilizers, allowing a reduction in the use of chemical fertilizers and a more sustainable production of tomatoes.

**Key words:** *Acinetobacter*, N<sub>2</sub> fixation, phosphorus and potassium solubilization, *Pseudomonas*, *Rahnella*, *Solanum lycopersicum*.

## INTRODUCTION

Nitrogen, P and K are essential macronutrients for plant growth and crop yield. However, they are not readily available to plants (Meena et al., 2014). The largest reservoir of N is found in the form of atmospheric N (78%), in a form biologically unavailable to plants (Hartono et al., 2016). Phosphorous in soil is present as P in solution, insoluble organic P and insoluble inorganic P, soils are often high in insoluble mineral phosphates but deficient in soluble orthophosphate, which is essential for the growth of most plants (Hariprasad and Niranjana, 2009). The concentration of soluble K in soil is usually very low (1% to 2%) since K is mainly present as insoluble rocks, silicate minerals and other deposits, although

it is not always available to plants (Parmar and Sindhu, 2013). Rhizobacteria are able to convert unavailable forms of nutrients into available ones for subsequent uptake by the plant (Meena et al., 2014).

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that are able to colonize rhizosphere and to enhance plant growth by means of a wide variety of mechanisms. Diverse species PGPR have been characterized as N fixers, P solubilizers and capable to solubilize mineral K from unavailable forms. N<sub>2</sub> fixation occurs when molecular N (N<sub>2</sub>) is converted into ammonia (NH<sub>3</sub>) by the enzyme complex nitrogenase, which consists of two metalloproteins, the molybdenum-iron protein (Protein 1) and the iron-protein (Protein 2). Nitrogenase requires the collaboration of two other proteins: ferredoxin and avodoxin, which act as electron donors and natural reducers of nitrogenase. The electrons are transported to the nitrogenase by ferredoxin and reach the iron-protein. This activates the Mo-Fe-protein and the reduction of N<sub>2</sub> occurs, being then fixed as an amino compound (Hoffman et al., 2014). Solubilization of inorganic P occurs by secretion of organic and inorganic acids, such as citric, lactic, succinic and gluconic acids. Hydroxyl and carboxyl groups of acids chelate cations (Ca<sup>2+</sup>) and lower the pH by releasing the phosphates (Behera et al., 2014). Potassium-solubilizing bacteria (KSB) can produce several types of organic acids; succinic, citric, gluconic, α-ketogluconic and oxalic, and inorganic; acetate, citrate, oxalate that when released to the rhizosphere cause the fixed K of the minerals to be released and dissolved by processes of acidolysis, chelation and complexolysis. The solubilization of K varies with the nature of the minerals (motmorillonite, kaolinite, K-feldspar, muscovite, biotite, illite, orthoclases) that contain K and aerobic conditions (Meena et al., 2015).

Of the large number rhizobacteria, the following genera have shown potential as biofertilizers or growth promoters: *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Acinetobacter*, *Beijerinckia*, *Derrxia*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, *Enterobacter*, *Bacillus*, *Rahnella*, *Alcaligenes*, *Klebsiella*, *Lysobacter* and *Paenibacillus*. The use of PGPR has proven to be an environmentally sound way of increasing crop yields by facilitating plant growth. It can also reduce production costs and the environmental impact associated with chemical fertilization (Majeed et al., 2018).

Several PGPR have been isolated from different soils and crops in Central Chile and have shown that they can promote root and aerial growth, and increase nodule formation in legumes (Gerding et al., 2017; Sepúlveda-Caamaño et al., 2018; Cedeño-García et al., 2018). The aim of this study was to select rhizobacteria capable of improving NPK availability and promoting tomato growth.

## MATERIALS AND METHODS

### Selection of rhizobacteria for N<sub>2</sub> fixation, and solubilization of P and K

Fifteen bacterial isolates from the Microorganism Collection of the Faculty of Agronomy, Universidad de Concepción, Chillán, Chile, were studied. These isolates had been formerly collected from the rhizosphere of nine major agricultural crops in Chile, grown in different soils of the Maule, Ñuble and Biobío Regions (Table 1). The isolates were reactivated in standard nutrient agar.

The bacteria were evaluated under laboratory conditions for (1) N<sub>2</sub> fixation, (2) P inorganic solubilization, and (3) K solubilization of minerals. Using the specific culture media Burk's, N-free medium (Hartono et al., 2016), Pikovskaya (PVK) (Hariprasad and Niranjana, 2009) and modified Aleksandrov medium (MAMs), waste mica was used biotite (11% K) and K-feldspar (14% K) (Meena et al., 2015), respectively.

**Capacity of the isolates to fix N<sub>2</sub> and solubilize P and K.** The screenings were carried out with three replicates for each of the isolates. Petri dishes with Burk's and PVK were incubated at 25 °C for 72 h, while MAMs was incubated at 28 ± 1 °C for 7 d. The ability of the isolates to fix N<sub>2</sub> and solubilize P and K was evaluated as follows: N<sub>2</sub> fixation was assessed by measuring the growth of the bacterial colony in N-free medium (Burk's). A numerical scale for growth in Burk's culture medium (GBCM) was used, where 3 was considered abundant growth; 2, good growth; 1, moderate growth; and 0 (zero), no visible growth. To evaluate P and K solubilization in PVK and MAMs, the diameter (mm) of the solubilization halo (translucent zone produced around each colony) was measured using a digital vernier (Hariprasad and Niranjana, 2009; Meena et al., 2015). Microphotographs of feldspar-K solubilization in MAMs media were taken from plates with and without bacterial inoculum. The microphotographs were taken at the Center for Mineralogical Studies CEM Geoatacama, with a scanning electron microscope (VEGA3, TESCAN, Kohoutovice, Czech Republic).

**Table 1. Host plant, place of collection and production of NPK of the rhizobacteria.**

Soil type	Geographic origin	Host plant	Location in the root	Strain	N <sub>2</sub>		P		K	
					GBC	NH <sub>4</sub> <sup>+</sup>	PSH	PO <sub>4</sub>	KSH	K
Andisol	36°37'21" S, 71°51'53" W	<i>Capsicum annuum</i> L. <i>Lens culinaris</i> Medik. <i>Apium graveolens</i> L.	Exorhizosphere	Aj-19b	3	mg L <sup>-1</sup> 1.4	mm 5.1	mg L <sup>-1</sup> 109.2	mm 0.0	mg L <sup>-1</sup> 24.0
			Endorhizosphere	Ltj-62	2	0.2	5.4	120.5	3.8	29.0
			Exorhizosphere	Ap-3bF	3	0.1	3.4	106.7	1.1	15.0
			Exorhizosphere	Ap-77a	2	0.1	0.0	160.4	5.1	0.0
		<i>Vicia faba</i> L. <i>Solanum lycopersicum</i> L.	Endorhizosphere	Hb-142	3	0.7	3.8	225.1	2.7	0.0
			Endorhizosphere	Tmt1-107	3	0.6	4.1	308.6	5.6	0.0
			Exorhizosphere	Ls-C54	3	0.0	1.2	155.0	4.3	37.0
		<i>Lactuca sativa</i> var. <i>capitata</i> L. <i>Triticum aestivum</i> L.	Endorhizosphere	Ls-C21	1	0.0	6.1	144.7	5.4	30.0
			Endorhizosphere	Ls-C58	1	0.1	5.5	241.2	3.2	25.0
			Exorhizosphere	T-35b	3	0.1	1.9	133.5	3.8	20.0
Ultisol	36°45'78" S, 72°04'8" W	<i>S. lycopersicum</i>	Rhizoplane	T-06	3	0.2	0.0	126.4	5.4	12.0
			Exorhizosphere	Tmt-22	2	0.1	0.9	181.9	4.8	18.0
			Exorhizosphere	Tmt-16	1	1.7	5.5	84.4	1.5	5.0
			Exorhizosphere	Tmt-32	2	0.0	7.5	144.4	4.6	26.0
Alfisol	35°57'20" S, 72°17'9" W	<i>Medicago sativa</i> L.	Exorhizosphere	GE-11	3	0.3	1.0	165.8	3.6	0.0

GBC: Growth in Burk's culture medium, 3 = abundant, 2 = good, 1 = moderate. PSH: Solubilization halo in Pikovskaya medium (PVK). KSH: Solubilization halo in modified Aleksandrov medium (MAMs).

**Determination of NPK availability in solution.** Burk's, PVK and MAMs liquid media were used to quantify the amount of fixed N and solubilized P and K by the bacterial strains selected. Liquid media were inoculated with each isolate and incubated in a rotary shaker (150 rpm) at 25 °C for 3 d for Burk's and PVK, and MAMs at 28 ± 1 °C for 7 d.

For each evaluation, 20 mL of bacterial suspension prepared with 100 µL of bacterial culture (optical density (OD) 600 nm = 1.0) suspended in 0.89% NaCl (w/v) were placed in 50 mL sterile falcon tubes. After incubation, the samples were centrifuged (Centrifuge 5804 R, Eppendorf, New York, USA) for 10 min at 11000 rpm; the supernatant was removed with a syringe with a sterile 0.45-µ filter and placed in 16 mL sterile test tubes. Each filtered sample was used to determine NH<sub>4</sub><sup>+</sup>, phosphate (PO<sub>4</sub>) and K in solution.

The production of N-NH<sub>4</sub><sup>+</sup> (mg L<sup>-1</sup>) by the selected isolates was assessed by direct Nesslerization (Vyas et al., 2010). For each sample, a volume of 2 mL of filtrate was taken. Then 2 mL Nessler reagent and 3 mL NaOH (3 M) were added, vortexed and allowed to stand for 20 min before reading in the spectrophotometer (Optizen POP-Bio, Mecasys, Daejeon, Korea) at 490 nm. The calibration curve was constructed with N-NH<sub>4</sub><sup>+</sup> at 0, 2, 5, 10, 20 and 30 mg L<sup>-1</sup>.

The solubilization of P-PO<sub>4</sub> (mg L<sup>-1</sup>) was assessed by colorimetry with the molybdenum blue method using C<sub>6</sub>H<sub>8</sub>O as reductant, with modifications (Behera et al., 2014). Samples of the filtrate were diluted 10 times. Volumes of 2 mL of the diluted sample were placed in 100-mL flasks. Then 43 mL distilled water and 5 mL reagent were added to determine phosphate. The mixture was homogenized in an orbital stirrer (Super-Mixer, Lab-Line Instruments, Melrose Park, Illinois, USA) for 5 s and allowed to stand for 30 min to observe color changes to azure blue. The readings of the samples were performed on a spectrophotometer (UVE 054108, Helios Epsilon, Thermo Scientific, Waltham, Massachusetts, USA) at 720 nm. The calibration curve was made with a phosphate standard solution at 1000 mg P-PO<sub>4</sub> L<sup>-1</sup>, at concentrations of 0.04, 0.08, 0.16, 0.24, 0.32, 0.40 and 0.48 mg P-PO<sub>4</sub> L<sup>-1</sup>.

The solubilization of K (mg L<sup>-1</sup>) was determined by atomic emission spectrometry of air-acetylene flame by direct aspiration (Zhang and Kong, 2014). The readings of the samples were performed at a wavelength of 766.5 nm. The calibration curve was performed with a standard solution of 1000 mg L<sup>-1</sup> in the following concentrations 0.0, 2.5 and 5.0 mg L<sup>-1</sup>.

#### Identification of selected bacterial isolates

Cells were prepared for polymerase chain reaction (PCR), by suspending 100 µL of bacterial culture in 900 µL nuclease-free water and centrifuging at 5000 g for 5 min. The supernatant was removed, and the cell pellet was

resuspended in 1 mL nuclease-free water. This procedure was repeated twice and finally the cell concentration was standardized at an OD 600 nm = 0.4 (CE 1020, Cesi Instruments, Cambridge, UK). Finally, the samples were subjected to a temperature shock at 65 °C for 5 min and then at -20 °C for 5 min (Guiñazú et al., 2013).

The 16S rRNA partial gene was amplified through PCR using the universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction mix consisted of 2 µL of cell suspension, 2 U Taq DNA polymerase (GoTaq, Promega, Madison, Wisconsin, USA), 0.5 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 1X GoTaq buffer solution, 0.2 mM dNTPs and ultrapure water Hyclone (Thermo Scientific) for a final volume of 40 µL. The PCR conditions were the following: one initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 7 min. The amplification of the gene was verified through electrophoresis in agarose gel 1% (w/v), stained with GelRed (Biotium, Hayward, California, USA) at 90 V for 45 min (Sepúlveda-Caamaño et al., 2018).

The PCR products were purified and sequenced at Macrogen Inc. (Seoul, Korea; <http://dna.macrogen.com/eng>). The chromatograms obtained were analyzed and edited in the GeneTool Lite 1.0 (2000) software. The phylogenetic tree was constructed using the Neighbor-Joining algorithm using MEGA 7 software (Tamura et al., 2004). Type strains sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov>).

### Endophytic capacity of selected bacterial isolates

Fragments 3 cm long were taken from the distal ends of the roots, stem and leaves of the inoculated plants. Then these sections were washed in distilled water and disinfected superficially in 70% ethanol for 2 min, followed by immersion in 1% hypochlorite for 30 s, and four washes in sterile distilled water. Subsequently, the fragments were cut into 1 cm pieces and cultured in standard nutrient agar plates, incubated for 48 h at 25 °C (Parsa et al., 2013). The bacterial colonies developed from the ends of the cuts were re-isolated on standard nutrient agar and subject to the same growth conditions mentioned above. Each treatment was established in triplicate for molecular analysis.

The identity of the isolates obtained was assessed at the strain level by molecular fingerprinting using the primer BOX A1R (5'-CTA CAA CGG GCT GAC GGC GAC G-3') according to the methodology used by Guiñazú et al. (2013). The PCR reaction mix contained 5.0 µL of cell template, 0.3 µL Gotaq polymerase (Promega) (5 U µL<sup>-1</sup>), 5.0 µL A1R primer (10 µM) 0.5 µL dNTPs (10 mM), 5.0 µL Gotaq buffer (5×) solution, 1.5 µL MgCl<sub>2</sub> (25 mM) and 7.7 µL ultrapure water, making a total of 25 µL. The PCR conditions were: 95 °C for 7 min; then 35 cycles at 94 °C for 1 min; 52 °C for 1 min and 72 °C for 8 min; and finally, 72 °C for 16 min with amendments in the time of PCR runs. The PCR products were analyzed by electrophoresis in 2% (w/v) agarose gels with 2.5 mL<sup>-1</sup> µL Gel Red (10 000× in DMSO, Biotium) in a 1× TAE buffer solution (40 mM Tris- acetate, 1 mM EDTA, pH 8.0) at 50 V for 3 h. Bands were visualized in a UV transilluminator.

### Effects of the selected bacterial isolates in plant and soil

The capacity of bacteria to promote plant growth in tomato 'Cal Ace' was evaluated. Plants were grown in seedlings using soil of the following characteristics as a substrate: 7.0 mg kg<sup>-1</sup> available N (nitrates N-NO<sub>3</sub> 4.90 mg kg<sup>-1</sup> and ammonium N-NH<sub>4</sub><sup>+</sup> 2.10 mg kg<sup>-1</sup>), 19.20 mg kg<sup>-1</sup> available P, 40.90 mg kg<sup>-1</sup> available K, 5.66 pH in water and 4.18% organic matter. The soil was classified as Isotic, mesic Dystric Xerosamments belonging to the Arenales series.

Three experiments were carried out, one for each type of selected bacteria; N<sub>2</sub> fixing bacteria (3 NFB strains + 1 negative control without inoculation), P solubilizers (4 PSB strains+ 1 negative control) and K solubilizers (5 KSB strains + 1 negative control). A completely randomized design was used with three replicates. Each experimental unit consisted of 27 plants.

The experiments were carried out in a phytotron with high-pressure sodium steam lamps (400 W Gro-lux, Osram Sylvania, Danvers, Massachusetts, USA) providing a minimum photosynthetic photon flux density (PPFD) of 400-500 mol m<sup>2</sup> s<sup>-1</sup>. The temperature ranged from 21 to 27 °C during germination and from 21 to 24 °C during vegetative growth, with a RH between 60% and 80%. Planting and nutritional management of the plants was carried out based on Ojodeagua et al. (2008).

Seeds, soil and germination trays were disinfected prior to sowing. The seeds were disinfected with 5% hypochlorite (W/V) for 3 min and with 70% ethanol (w/v) for 1 min, followed by six washes in sterile distilled water. The soil was disinfected with steam at an initial temperature of 100 °C and then maintained at 82 °C for 30 min. The germination trays were disinfected with 10% hypochlorite (w/v).

**Plant inoculation.** Plants were inoculated twice: at emergency stage and 15 d after emergency. Bacterial concentration for each strain was standardized at  $10^7$  CFU mL<sup>-1</sup> in 1.0% sucrose (v/v) determined through absorbance ( $\lambda = 600$  nm). For inoculation, 1 mL standardized bacterial suspension was placed aseptically in the root zone of each plant.

**Plant growth promotion.** At day 35 after planting, plants were removed from the soil and their roots carefully washed. The length (cm) and fresh and dry weight (g) of both roots and aerial shoots were evaluated. Dry weight was determined after roots and shoots were dried in a stove until reaching constant weight (70 °C/48 h).

**NPK determination in plant tissue and soil.** NPK were determined through soil analysis methods of the Commission for Standardization and Accreditation of the Chilean Society of Soil Science. In plant the N analysis was carried out by the digestion method and colorimetric determination; in soil inorganic N was determined by colorimetry, nitrates via nitration of salicylic acid, and ammonium using Nessler reagent. Phosphorous content was determined by calcination and colorimetric determination of the phospho-vanadomolybdate, and K content was determined through calcination and atomic emission spectrometry.

### Statistical analysis

Data obtained in laboratory were subjected to cluster analysis (standardized data) using Euclidean metric as a measure of distance. The defined clusters and the bacterial isolates of the best cluster(s) were subjected to a nonparametric variance analysis with the Kruskal Wallis test and a comparison of means test. Data obtained each trial in plant, completely randomized design with three replicates, were subjected to ANOVA and means were compared through the Tukey test. Analysis were realized with a probability of  $p \leq 0.05$  using the InfoStat/E 2013 software.

## RESULTS AND DISCUSSION

### Selected rhizobacteria for N<sub>2</sub> fixation, solubilization of P and K

**N<sub>2</sub> fixation and ammonium production (N-NH<sub>4</sub><sup>+</sup>).** The 15 isolates evaluated had growth of bacterial colony in Burk's agar medium; 8 isolates had abundant growth, 4 had good growth and 3 had moderate growth. Determination of N-NH<sub>4</sub><sup>+</sup> in Burk's liquid medium showed that 12 out of the 15 strains made NH<sub>4</sub><sup>+</sup> available (Table 1). These bacterial strains probably fixed the N<sub>2</sub> contained in the air of the falcon tubes and used it for their metabolism. N<sub>2</sub> fixation occurs when molecular N (N<sub>2</sub>) is converted into ammonia (NH<sub>3</sub>) by the enzyme complex nitrogenase, which consists of two metalloproteins, the molybdenum-iron protein (Protein 1) and the iron-protein (Protein 2). Nitrogenase requires the collaboration of two other proteins: ferredoxin and avodoxin, which act as electron donors and natural reducers of nitrogenase. The electrons are transported to the nitrogenase by ferredoxin and reach the iron-protein. This activates the Mo-Fe-protein and the reduction of N<sub>2</sub> occurs, being then fixed as an amino compound (Hoffman et al., 2014). The process of N<sub>2</sub> fixation varies between different bacterial genera. Most of biological N fixation is carried out by the activity of molybdenum nitrogenase, which is found in all diazotrophs (Carvalho et al., 2014).

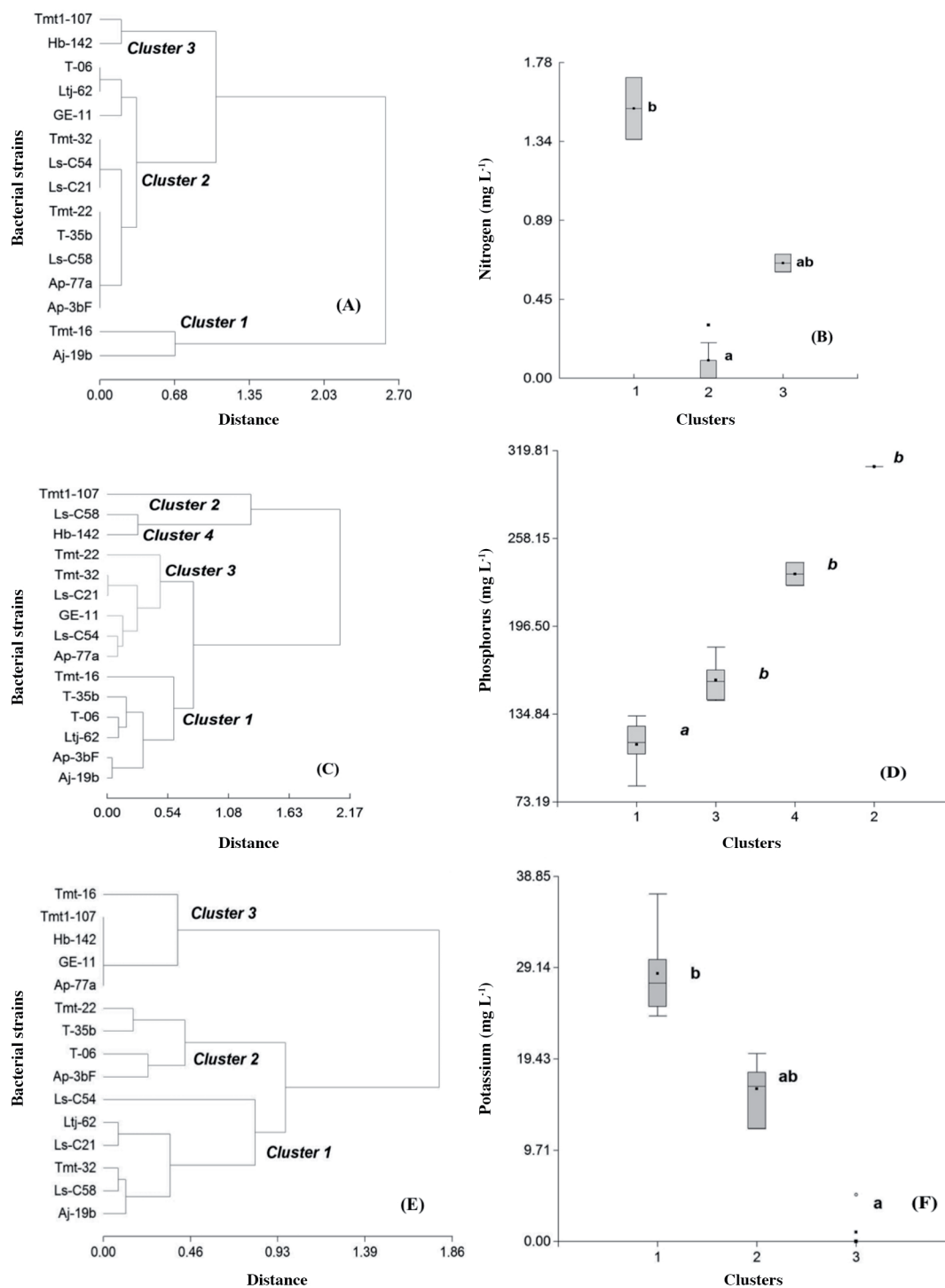
The concentrations of N-NH<sub>4</sub><sup>+</sup> produced ranged from 0.2 to 1.7 mg L<sup>-1</sup> (Table 1). Studies on asymbiotic N<sub>2</sub> fixing bacteria (NFB) have reported higher levels of NH<sub>4</sub><sup>+</sup> than the ones on this work, with concentrations of 5.17 mg L<sup>-1</sup> by *Azotobacter* sp.; 3.65 mg L<sup>-1</sup> by *Azotobacter vinelandii* DSM 2289; 16.63 to 6.89 mg L<sup>-1</sup> by *Azotobacter vinelandii* (GMA6 and GMA9); 4.68 mg L<sup>-1</sup> by *Azospirillum* spp.; 1.948 mg L<sup>-1</sup> by *Azospirillum brasilense* DSM 1224; 10.34 mg L<sup>-1</sup> by *Brevibacillus formosus* (GMA3); and 5.88 mg L<sup>-1</sup> by *Stenotrophomonas* sp. (GMA1) (Hartono et al., 2016).

For the selection of NFB, the 15 strains were grouped into three clusters (CC = 0.96): CL1, CL2 and CL3 (Figure 1A) CL1 presented mean NH<sub>4</sub><sup>+</sup> concentrations of 1.53 mg L<sup>-1</sup>, producing 1.42 mg L<sup>-1</sup> more than CL2 (0.11 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>) and 0.88 mg L<sup>-1</sup> more than CL3 (0.65 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>) (ANOVA,  $P = 0.002$ ). However, CL3 did not show significant differences with respect to CL1 and CL2 (Figure 1B). Therefore, an ANOVA was performed between CL1 and CL2 isolates: Aj-19b (1.35 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>), Hb-142 (0.70 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>), Tmt1-107 (0.60 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>), Tmt-16 (0.70 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>). As no differences were found in terms of NH<sub>4</sub><sup>+</sup> production ( $p = 0.40$ ), all isolates were selected for the plant trials.

The four bacterial strains selected as NFB were isolated from the following crops: chili, Aj-19b; faba bean, Hb-142; tomato, Tmt1-107 and Tmt-16. Nitrogen-fixing bacteria have the ability to develop different types of root associations with different plant species (Carvalho et al., 2014).



**Figure 1. Phenogram of the cluster analysis of the 15 rhizobacteria, and comparison between clusters: (A) (B)  $\text{NH}_4^+$  production; (C) (D) P solubilization; (E) (F) K solubilization.**



Average - average linkage, Euclidean distance. Means with the same letter are nonsignificantly different (p > 0.05).

**Phosphate solubilization and P production (P-PO<sub>4</sub>).** Out of 15 isolates evaluated, 12 bacterial isolates presenting solubilization halos on the PVK-agar plate, with a halo from 0.9 to 7.5 mm (Table 1). Solubilization of inorganic P from Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> in PVK media occurs by secretion of organic and inorganic acids, such as citric, lactic, succinic and gluconic acids. Hydroxyl and carboxyl groups of acids chelate cations (Ca<sup>2+</sup>) and lower the pH by releasing the phosphates (Behera et al., 2014).

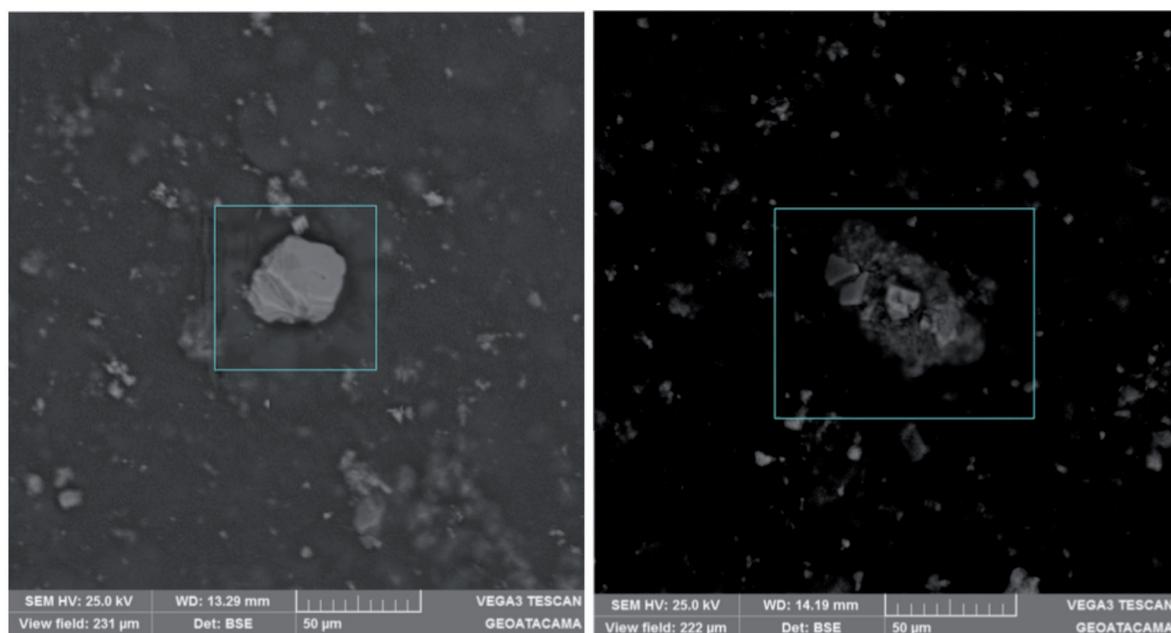
Determination of the available P in solution showed that the 15 strains evaluated were able to solubilize the phosphate of the tricalcium phosphate Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, ranging from 84.4 to 308.6 mg L<sup>-1</sup>. Several studies have reported that many isolates do not produce any visible halo zone on the agar plate, but they can solubilize several types of insoluble inorganic phosphate in liquid medium (Behera et al., 2017). This may be due to varying diffusion rates of different organic acids secreted by different microorganisms.

The 15 bacterial isolates were grouped into four clusters (CC = 0.83); CL1, CL, CL3 and CL4 (Figure 1C), consisting of 6, 1, 6 and 2 isolates with average P values of 113.45, 308.60, 158.70, 233.15 mg L<sup>-1</sup>, respectively. ANOVA showed significant differences (P = 0.0177) between CLs. The CL1 recorded the lowest P production in comparison with CL3, CL4 and CL2 (Figure 1D).

For the plant trials, the following isolates of CL2 and CL4 were selected: Tmt1-107 (308.60 mg P-PO<sub>4</sub> L<sup>-1</sup>), Ls-C58 (241.20 mg P-PO<sub>4</sub> L<sup>-1</sup>) and Hb-142 (225.10 mg P-PO<sub>4</sub> L<sup>-1</sup>). These isolates solubilized P in larger amounts than those reported in other studies by Ehsan et al. (2016) that determined P solubilization from tricalcium phosphate by species of the genus *Pseudomonas*, *P. beteli* and *P. lini*, which solubilized phosphate in a range from 18.30 to 147.53 µg mL<sup>-1</sup>. There are reports that indicate that the use of PSB in co-composting of urban waste (95%) + phosphate rock (5%) can generate up to 25 kg P t<sup>-1</sup> fresh weight ha<sup>-1</sup> (Naher et al., 2018).

**Potassium solubilization and production.** Out of 15 isolates evaluated 14 bacterial isolates solubilized K from K-feldspar in MAMs-agar, with a solubilization halo from 1.1 to 5.6 mm (Table 1). Solubilization of K occurs by secretion several types of organic acids; succinic, citric, gluconic, α-ketogluconic and oxalic, and inorganic; acetate, citrate, oxalate that when released to the rhizosphere cause the fixed K of the minerals to be released and dissolved by processes of acidolysis, chelation and complexolysis. Figure 2 shows the effect of a bacterium on K mica (K-feldspar) by breaking down the mineral and resulting in a 5% solubilization of the K contained in the feldspar. These KSB can produce several types of

**Figure 2. Microphotographs of feldspar-K solubilization in MAMs media from plates with and without bacterial inoculum.**



Unaltered K-feldspar, 14% K (left) compared to K-feldspar inoculated with illite/muscovite reaction boundary, 9% K, in modified Aleksandrov medium (MAMs) agar (right).

organic acids that possibly break down the structure of the micas to satisfy their  $\text{Si}^{4+}$  and  $\text{K}^+$  requirements in the culture medium within the plate, consequently reducing the pH of the medium.

Based on a  $\text{CC} = 0.82$  in the cluster analysis of the 15 bacterial isolates, three clusters were identified, CL1, CL2 and CL3 (Figure 1E), consisting of 6, 4 and 5 isolates with average K values of 28.5, 16.25 and 1.0  $\text{mg K L}^{-1}$ , respectively. ANOVA showed significant differences ( $P = 0.0018$ ) between CL1 and CL3; CL1 isolate showed the highest K-solubilizing capacity (Figure 1F). For the plant trials, six CL1 isolates were selected: Ls-C54 (37.0  $\text{mg K L}^{-1}$ ), Ls-C21 (30.0  $\text{mg K L}^{-1}$ ), Ltj-62 (29.0  $\text{mg K L}^{-1}$ ), Tmt-32 (26.0  $\text{mg K L}^{-1}$ ), Ls-C58 (25.0  $\text{mg K L}^{-1}$ ), and Aj-19b (24.0  $\text{mg K L}^{-1}$ ).

The six selected isolates showed K production ranging from 24.0 to 37.0  $\text{mg L}^{-1}$  (Table 1). Similar ranges were obtained by Parmar and Sindhu (2013) from 15 to 48  $\text{mg L}^{-1}$ , when quantifying the solubilization of K from mica powder by six bacterial strains. These values were lower than those reported for the strain *Bacillus mucilaginosus*, AS1.153 when solubilizing K in motmorillonite, kaolinite and K-feldspar, with values ranging between 90 and 140  $\text{mg K L}^{-1}$ . However, these values are higher than those obtained by Zhang and Kong (2014), where 17 isolates of KSB reached from 0.59 to 4.4  $\text{mg K L}^{-1}$ , and by *Pseudomonas azotoformans*, which solubilized 6.03  $\text{mg K L}^{-1}$  (Saha et al., 2016). The solubilization of K varies with the nature of the minerals (motmorillonite, kaolinite, K-feldspar, muscovite, biotite, illite, orthoclases) that contain K and aerobic conditions (Meena et al., 2015).

### Identification of the selected bacteria

For all selected strains, a 1200 bp fragment of the 16S rRNA gene was amplified. The phylogenetic tree based on 16S rRNA grouped the bacterial strains into three genera: *Pseudomonas*, *Rahnella* and *Acinetobacter* (Figure 3).

Of the strains within the *Pseudomonas* genus, Ltj-62 and Aj-19b were closely related to the type strain *P. brassicacearum* DBK11<sup>T</sup> (AF100321.1), with a similarity of 99.998%. Hb-142 was grouped with the type strain *P. marginalis* ATCC 10844<sup>T</sup> (AB021401.1), with a similarity of 100%. Tmt-16 was grouped with *P. gessardi*<sup>T</sup> (AF074384.1), with a similarity of 99.971%, and Tmt-32 and Ls-C21 were grouped with *P. koreensis* Ps 9-14<sup>T</sup> = LMG 21318<sup>T</sup> = KACC 10848<sup>T</sup> (NR 025228.1), with similarity scores of 99.998% and 100%, respectively.

Bacteria of the *Pseudomonas* genus have been extensively described as PGPR, and endophytes of several plants (Carvalho et al., 2014). Species such as *P. monteilii* and *P. mandelii* have been reported as NFB associated with rice plants (Habibi et al., 2014). In fact, *Pseudomonas* is a widespread bacterial genus, which presents the highest P solubilizing activity in rhizosphere soil (Bahena et al., 2015). *Pseudomonas azotoformans* has also been reported as K solubilizing bacteria (Saha et al., 2016).

Ls-C58 and Ls-C54 were identified as belonging to the *Acinetobacter* genus and were closely related to the type strain *A. calcoaceticus* DSM30006<sup>T</sup> (X81661.1), with similarity scores of 99.989% and 100%, respectively. *Acinetobacter calcoaceticus* has also been reported as PSB, and a Zn oxidizer (Rokhbakhsh-Zamin et al., 2011). *Acinetobacter* spp. has been described to have a high biological N fixation in sugarcane (Taulé et al., 2012). When associated with mycorrhizal fungi, this species has shown potential to contribute to remediating alkaline-saline soils contaminated with petroleum (Xun et al., 2015).

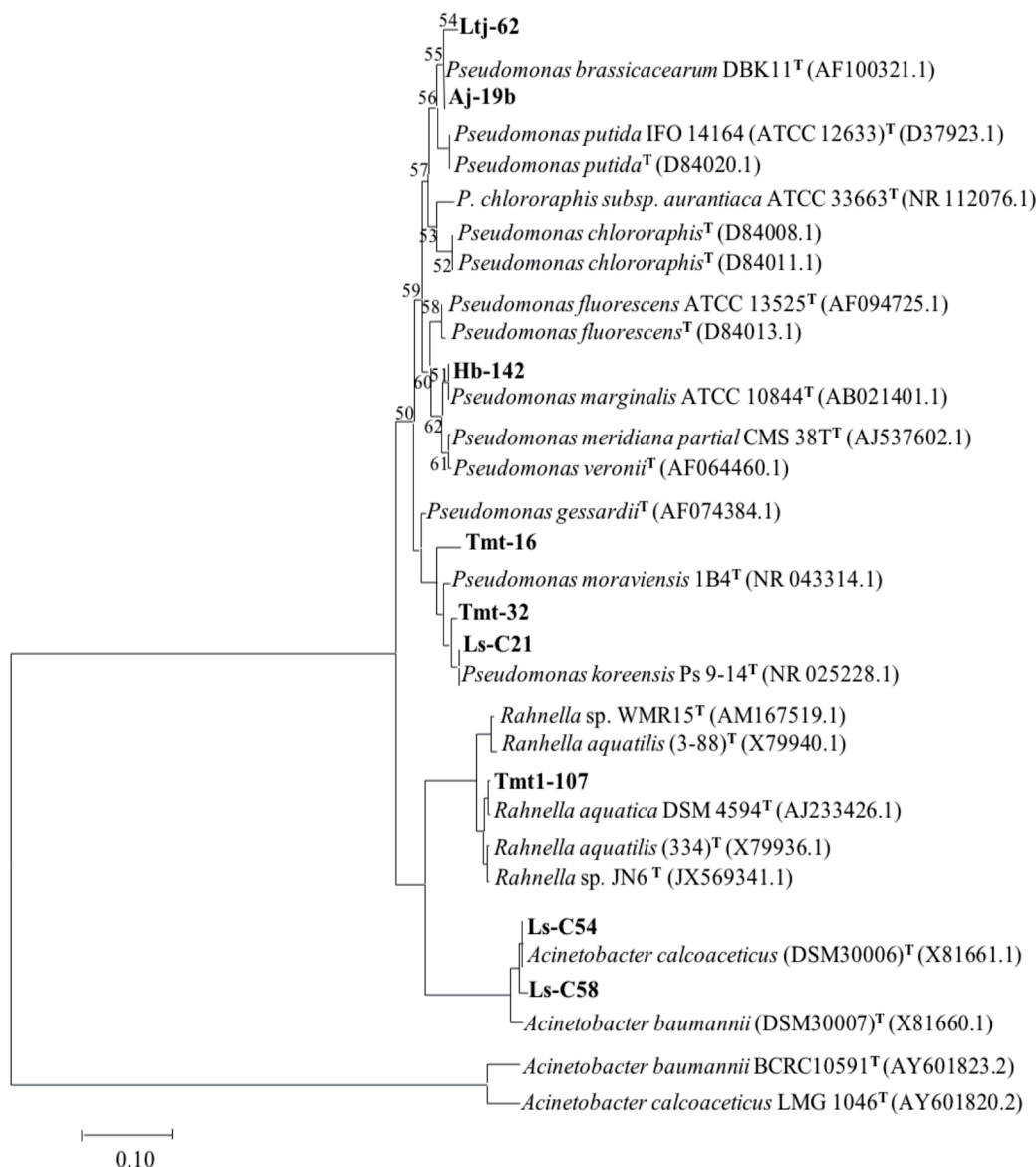
Tmt1-107 was identified as *Rahnella* sp., and as closely related to the type strain *R. aquatilis* 334T (X79936.1). *Rahnella* sp. is a bacterium described as PGPR in major agricultural crops. *Rahnella aquatilis* has been described as a PSB in the rhizosphere of *Eucalyptus* (Angulo et al., 2014). Furthermore, *Rahnella* sp., BIHB 783, has been described as an ammonia producing bacteria, and a P solubilizer, in barley, chickpea, pea, and maize (Vyas et al., 2010).

### Endophytic capacity of the selected bacteria

Endophytic bacteria colonize any region within the epidermis of the plant root, and they can reside in apoplastic intercellular spaces and the xylem vessel apoplast. These bacteria establish in less competitive niches that present better conditions for N fixation and assimilation of fixed N by the plant (Carvalho et al., 2014). Of the plants inoculated in the pot trials, 30 different strains were isolated based on the physical appearance of each colony. However, when the genetic fingerprint of these isolates was determined, results did not coincide with the band patterns of the original strain. Therefore, none of the eight strains selected as NFB, PSB and KSB had the ability to act as endophytes (Figure 4).



**Figure 3. Neighbor joining phylogenetic tree based on 16S rRNA sequencing of phylogenetic tree of *Pseudomonas* spp., *Rahnella* spp. and *Acinetobacter* spp. Bootstrap values are indicated on branches only when higher than 70. The type strains sequences in the phylogram were obtained from GenBank (accession number in parentheses). The bacterial strains studied are marked in bold.**

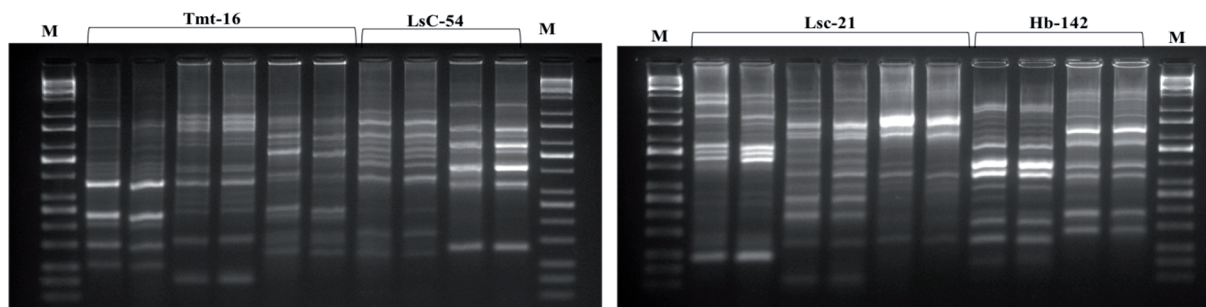


### Effect of the selected bacteria in plant and soil

**Effect of NFB.** Tmt1-107 *Rahnella aquatica*, and Hb-142 *P. marginalis* did not generate a positive effect on tomato plant growth. On the other hand, Tmt-16, *P. gessardii* (7.38 cm), increased root growth in 23.57% compared to the negative control (5.64 cm) ( $p = 0.05$ ). However, Tmt-16 was the strain that resulted in the lowest plant height, reaching 5.50 cm ( $p = 0.0047$ ), which was 2.17 cm lower than the control (7.67 cm). The results on higher root growth coincide with those of Naiman et al. (2009). The former reported an increase in root growth from 18% to 26% in tomato plants inoculated with *Bacillus subtilis* BEB-13bs (post-transplant) compared to the untreated control; the latter reported that there was a 40% increase in root biomass in wheat inoculated with *P. fluorescens*.

Tmt-16, *P. gessardii*, significantly increased N content in plant tissue in 2.60%, compared to the control and to strains Tmt1-107, Hb-142 ( $p = 0.0013$ ). This strain maintained the highest amount of N available in the soil, 2.95 mg kg<sup>-1</sup> ( $p = 0.0006$ ), and a higher content of N-NH<sub>4</sub><sup>+</sup> 1.95 mg kg<sup>-1</sup> ( $p = 0.0296$ ) (Table 2). This indicates that Tmt-16, *P. gessardii*,

**Figure 4. PCR amplification of the bacterial isolates from leaf and stem of the plants inoculated.**



From left to right, Tmt-16; band 1 and 2, control-strain, band 3 and 4 isolated from the leaf, band 5 and 6 isolated from the stem. LsC-54; band 1 and 2 control-strain, band 3 and 4 isolated from the leaf. LsC-21; band 1 and 2 control-strain, band 3 and 4 isolated from the leaf, band 5 and 6 isolated from the stem.

**Table 2. Effect of the N-fixing bacteria on N content in plant and N availability in soil.**

Isolates	N uptake	N available in soil	N-NH <sub>4</sub> <sup>+</sup> in soil	N-NO <sub>3</sub> in soil
	%			
Tmt-16	2.60a	2.95a	1.95a	1.00
Tmt1-107	1.19c	1.80b	1.25b	0.55
Hb-142	1.25c	1.70b	0.90b	0.90
Control	1.73b	1.50b	0.95b	0.55

Mean values with the same letter are nonsignificantly different ( $p > 0.05$ ).

is an N-fixing bacteria with potential use as biofertilizer or growth promoter. The change in root growth by Tmt-16, *P. gessardi*, resulted in greater root surface area for the uptake of nutrients and production, which also explains the higher N concentration in plant tissue. Studies in tomato indicate that inoculation with *Bacillus amyloliquefaciens* IN937a and *B. pumilus* T4 may increase plant growth and N availability. However, the effect of bacteria can vary and is influenced by factors such as N source, N rate, and soil fertility (Fan et al., 2017). In 2005, studies with the tomato ‘Amalia’ showed that applications of *Azospirillum* sp. (AzoFert) combined with 120 kg N increased yield in 11% in comparison to the control, resulting in a reduction of 30 kg N ha<sup>-1</sup> in the crop, which represented a decrease of 20% N application (Alfonso et al., 2005).

In cereals, the estimates of biological N<sub>2</sub> fixation by free-living bacteria generally range between 5 and 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> in enriched soil environments and plant residues (Roper and Gupta, 2016). The process of N<sub>2</sub> fixation varies between different bacterial genera. Most of biological N fixation is carried out by the activity of molybdenum nitrogenase, which is found in all diazotrophs (Carvalho et al., 2014). Of the large number of free-living N<sub>2</sub>-fixing bacteria, the following genera have shown potential as biofertilizers or growth promoters: *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia*, *Pseudomonas*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, *Derxia*, *Beijerinckia* and *Azotobacter*, *Enterobacter*, *Bacillus*, *Alcaligenes*, *Klebsiella*, *Lysobacter* sp. and *Paenibacillus polymyxa* (Majeed et al., 2018). Of all these genera, only the genus *Pseudomonas* was identified in this study.

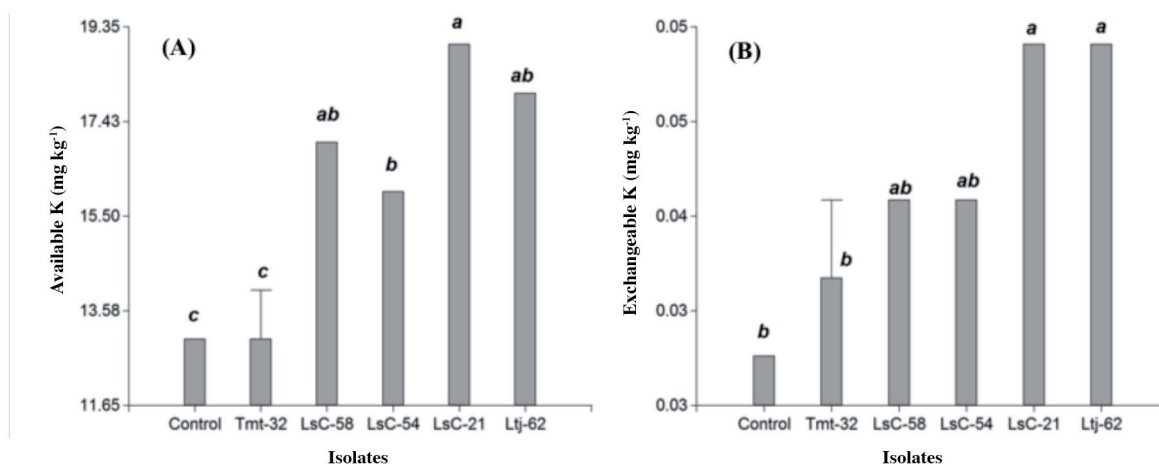
**Effect of PSB.** Hb-142 *P. marginalis*, LsC-58 *A. calcoaceticus* and Tmt1-107 *R. aquatilis* did not generate a positive effect on tomato plant growth. Regarding P solubilization, these strains did not generate an increase in the initial content of available P in the soil (19.2 mg kg<sup>-1</sup>). On the contrary, available P diminished, ranging from 14.8 to 16.8 mg kg<sup>-1</sup>. P content in the plant tissue ranged between 0.13% and 0.14% and was similar to that of the plants without bacterial inoculum (0.14%).

The effectiveness of PSB depends on the ability of the isolates to colonize the rhizosphere and maintain their biological activity, and on the types of metabolites produced and their release rate (Zhu et al., 2011). The performance of the PSB is influenced by with the nutritional richness and soil physicochemical properties such as organic matter, temperature and

soil pH (Alori et al., 2017). The results obtained indicate that the effect of the bacteria on plants and soil was strongly influenced by the soil pH (5.66). This is due to the fact that tomatoes grow best in slightly acid soil with a pH range from 6.0 to 7.5, and for the best replenishment of P in the soil, the pH should be in the range of 6 to 7. Therefore, the increase in soil acidity from 5.66 to 5.3 at the end of the experiment probably resulted in the formation of aluminum and iron phosphates, which led to symptoms of P deficiency in plants. On the other hand, most solubilizing microorganisms can solubilize calcium phosphate complexes and only some can solubilize aluminum or iron phosphate. Therefore, further studies are required to evaluate these PSB, which were selected in laboratory, in another type of soil and/or with slightly acid pH ranges.

**Effect of KSB.** The five bacterial strains classified as KSB neither promoted growth in tomato plants nor increased K content. The K solubilizing strains Ls-C21 *P. koreensis*, Ltj- 62 *P. brassicacearum* and LsC-58 *A. calcoaceticus* reached 17.0 to 19.0 mg kg<sup>-1</sup> available K ( $p = 0.0002$ ) and 0.04 to 0.05 mg kg<sup>-1</sup> exchangeable K ( $p = 0.0023$ ). Ls-C21, had the highest amount of available K 19.00 mg kg<sup>-1</sup>, maintaining 6 mg kg<sup>-1</sup> more than the soil without bacterial inoculation (Figure 5A, B). Sirajuddin et al. (2016) found that *B. pumilus* increases the solubility of K<sup>+</sup> and the antioxidant activity induced by tomato plants, which helps plants maintain their yields under stress conditions. About 90% of K exists in forms of insoluble rock and silicate minerals, but soluble K is generally very low in the soil (Parmar and Sindhu, 2013). In fact, K deficiency has become a major limitation for crop production. Inadequate K nutrition negatively affects root development, seed production and yield (Zhang and Kong, 2014). These results indicate that these three strains have potential as K solubilizers and can be an alternative to maintain K levels in the soil.

**Figure 5. Effect of K solubilizing bacteria on the soil: Available (A) and exchangeable K (B).**



Means with the same letter are nonsignificantly different ( $p > 0.05$ ).

## CONCLUSIONS

Four bacteria strains were selected for their capacity to fix N<sub>2</sub>, three for their ability to solubilize P, and six for their capacity to solubilize biotite and K-feldspar, isolated from tomato, lentil, chili pepper, faba bean and lettuce crops in Andisol and Alfisol soils.

These bacteria selected in laboratory, four bacteria were selected because of their capacity to maintain levels of N and K available to plants, in an Ultisol with a low content of available N and K. Three of them belong to the genus *Pseudomonas*: Tmt-16, *P. gessardi*; Ls-C21, *P. koreensis*; Ltj-62 *P. brassicacearum* and one corresponds to genus *Acinetobacter*: LsC-58, *A. calcoaceticus*, collected from an Ultisol and Andisol from tomato, lettuce and lentil crops. Tmt-16 increased root growth, which is a quality parameter in the production of tomato seedlings. P solubilizing strains did not show a positive effect on tomato plant growth or increase in available soil P.

These four endemic rhizobacteria of Chile can be potentially used as biofertilizers, allowing a reduction in the use of chemical fertilizers and a more sustainable production of tomatoes.

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