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CHILEANJAR
CHILEAN JOURNAL of AGRICULTURAL RESEARCH

Plant growth promoting rhizobacteria with ACC deaminase activity isolated from Mediterranean dryland areas in Chile: Effects on early nodulation in alfalfa

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Received: 7 May 2018; Accepted: 11 June 2018; doi:10.4067/S0718-58392018000300360

ABSTRACT

Alfalfa (*Medicago sativa* L. subsp. *sativa*) has emerged as an alternative crop for Mediterranean dryland areas in Chile. These soils show deficient nutritional levels affecting legume nodulation and N fixation through symbiosis with *Ensifer meliloti*. The objective of this work was to select plant growth promoting rhizobacteria (PGPR) associated with alfalfa and to evaluate their effects in plant growth and nodulation. Bacterial strains were isolated from the rhizosphere of alfalfa cultivated in dryland soil in Mediterranean areas of Chile. The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, production of indole acetic acid (IAA), phosphate solubilization and the compatibility with *E. meliloti* was assessed for each strain. The selected bacterial strains were identified by the amplification of the 16S rRNA gene. Twelve of the 32 isolated strains were compatible with *E. meliloti* as they did not show signs of antagonism. Eight strains solubilized phosphate, 12 produced IAA ranging from 13.71 to 26.22 mg L^{-1} and only the GN-8 isolate reported ACC deaminase activity. In the plants, the nodulation was greater when the strain with ACC deaminase and *E. meliloti* were co-inoculated both in laboratory and greenhouse conditions ($P < 0.05$). The selected bacteria GN-2 and GN-8 belonged to the genus *Bacillus*, while GN-4 to *Pseudomonas*. In conclusion, PGPR collected in marginal soils of Mediterranean areas of Chile showed ACC deaminase activity, IAA production and phosphate solubilization. The co-inoculation with *E. meliloti* and *Bacillus* sp. GN-8 allowed earlier nodulation in alfalfa.

Key words: ACC deaminase, alfalfa, *Medicago sativa*, nodulation, PGPR, phosphate solubilization, indole acetic acid.

INTRODUCTION

Alfalfa (*Medicago sativa* L. subsp. *sativa*) is the most important and widely grown forage legume in the world (Bouton, 2012). Alfalfa establishes a symbiotic mutualistic relationship with the bacteria *Ensifer* (*Sinorhizobium*) *meliloti*, through the formation of a root structure called nodule where N fixation occurs (Pini et al., 2012). Nodulation in legumes is a process regulated by biochemical signals between plant and bacteria through flavonoid aromatic compounds secreted by the plant, and bacterial compounds known as Nod Factors (Ji et al., 2017). Early nodulation is the key for a better

establishment of this symbiotic relationship to ensure increased N fixation and plant biomass production (Pini et al., 2012). Alfalfa can achieve fixation levels of 120-800 kg N ha^{-1}. However, in environments with adverse conditions for the rhizobia such as the Mediterranean dryland area of Chile, nodulation and symbiotic effectiveness in alfalfa may be drastically reduced (del Pozo et al., 2017). Nodulation is inhibited by the increase of ethylene in response to defense signals from interactions of plants with other microorganisms and very often by periods of droughts and soil salinity (Shahzad et al., 2010; Barnawal et al., 2014).

Significant improvements in nodulation have been reported through inoculation with plant growth promoting rhizobacteria (PGPR) able to produce indole acetic acid (IAA) and with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Rashid et al., 2012; Sepúlveda-Caamaño et al., 2018). The presence of ACC deaminase-producing bacteria in the root system reduces ethylene biosynthesis and increases the number of nodules, and produces changes in root architecture by promoting the development of lateral roots (Shahzad et al., 2010). This is because 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme degrades and consumes the ethylene precursor ACC or inhibits the enzyme ACC synthase (Hardoim et al., 2008). When the ethylene precursor ACC is broken by the enzyme ACC deaminase, it is converted to alpha-ketobutyrate and ammonium, providing the bacteria with a source of N (Penrose and Glick, 2003). Co-inoculating legumes with bacteria with ACC deaminase activity and rhizobia, can improve nodulation by up to 40%, therefore enhancing N fixation (Ma et al., 2004; Shahzad et al., 2010; Glick, 2014).

The objectives of this research were to select PGPR from alfalfa rhizosphere with ACC deaminase activity, capable of producing IAA and solubilizing phosphate and to evaluate the effect of the co-inoculation *Ensifer meliloti*-PGPR on early nodulation in alfalfa plants.

MATERIALS AND METHODS

Collection and isolation of bacteria

Soil and alfalfa plants (10 samples) were collected from a rainfed area in Mediterranean central Chile, Cauquenes (35°57'20.63" S, 72°17'9.14" W), an area characterized by granitic soils (Ultic Palexeralf), a soil pH of 7.0, 1.6% organic matter content, 2.3 mg kg^{-1} available mineral N, 12 mg P kg^{-1} and 185 mg K kg^{-1} in the top 20 cm. To isolate bacteria from the endorhizosphere, the protocol described by Sepúlveda-Caamaño et al. (2018) was used, where 1 g root was disinfected by immersion in 70% (v/v) ethanol for 30 s and rinsed in sterile distilled water for 1 min. To isolate bacteria from the exorhizosphere, 1 g soil firmly attached to the root was used. Samples of disinfected roots and soil were separately macerated in sterile mortars in 100 mL phosphate-buffered saline (PBS) solution (0.9% NaCl (v/v), 2.9 mM KH_2PO_4, 7.1 mM K_2HPO_4, pH 7.2) and stirred for 2 h at 150 rpm. The suspensions were diluted to concentrations of 10^{-3}; from each dilution, 0.1 mL was inoculated in Petri dishes with standard nutrient agar medium (Merck, Kenilworth, New Jersey, USA) and incubated at 25 °C for 48 h. Isolated colonies with distinctive morphological characteristics were selected and re-isolated on standard nutrient agar to obtain pure cultures. The isolates were cryopreserved at -80 °C in 20% glycerol (Oskouei et al., 2010).

PGPR compatibility with *Ensifer meliloti*

The capacity of the plant growth promoting rhizobacteria (PGPR) isolates to grow together with the alfalfa specific inoculant *E. meliloti* strain AG-06 was tested. For this purpose, the rhizobacterial strains, in addition to AG-06 were placed in 5 mL of sterile saline solution (0.98% w/v) and the optical density was adjusted to 600 nm wavelength (OD_{600}) at 0.1. The suspensions were inoculated into nutrient agar plates and superficially distributed with a glass rod and were incubated at 25 °C. An agar disc showing bacterial growth was placed on the plates inoculated with *E. meliloti*, kept in incubation at 25 °C for 24 h prior to evaluation. When *E. meliloti* was not able to grow around the bacterial disc, the strain was discarded from future experiments. Only the isolates that were compatible with *E. meliloti* strain AG-06 were selected for the following experiments.

ACC deaminase activity of PGPR selected

An aliquot of 100 µL of suspended cells (10^7 CFU mL^{-1}) of the selected strains, were inoculated onto 3 mL nutrient broth and incubated for 24 h at 200 rpm at a constant temperature of 25 °C. Then 1 mL of cell suspension was transferred to

microtubes and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was extracted and washed twice in Dworkin-Foster (DF) minimal salts medium (Dworkin and Foster, 1958) and centrifuged at 8000 rpm for 10 min at 4 °C. The cell pellet was then suspended in 500 µL DF minimal media and incubated in a rotary shaker at 200 rpm and 25 °C for 24 h. Bacterial strains were inoculated onto three different media: Plates with 30 mL DF agar; DF agar with 1.5% 1-aminocyclopropane-1-carboxylic acid (ACC), and DF agar with $(NH_4)_2SO_4$ as N source (Penrose and Glick, 2003). A droplet of 5 µL of each strain was placed on each media. Strains with ACC deaminase activity: *Pseudomonas* sp. 8R6 wild type (WT) and *Pseudomonas fluorescens* YsS6 WT were included as positive controls; and two ACC deaminase mutant (acds) mutant strains, 8R6 acds and YsS6 acds, were included as negative controls (Rashid et al., 2012). Colony growth was observed daily for 5 d.

Phosphate solubilization of PGPR selected

Phosphate solubilization was evaluated according to Chaiharn and Lumyong (2011). The isolates were grown in standard nutrient broth (Merck), shaken for 48 h at 150 rpm at 25 °C. Plates with Pikovskaya agar (glucose 10 g, $Ca_3(PO_4)$ 25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, yeast extract 0.5 g, $MnSO_4$ traces, $FeSO_4$ traces, 15 g agar, 1000 mL distilled water, pH 7-7.5) were inoculated with 4 µL pure bacterial culture. They were incubated at 25 °C for 48 h and the formation of the transparent halo was observed in each colony.

To quantify the solubilized phosphate, bacterial isolates capable of solubilizing phosphate were inoculated in sterile Falcon tubes with 10 mL Pikovskaya liquid medium (glucose 10 g, $Ca_3(PO_4)$ 25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, yeast extract 0.5 g, $MnSO_4$ traces, $FeSO_4$ traces, distilled water 1000 mL, pH 7.3). In addition, a sample of Pikovskaya liquid medium without inoculation was used as control. Samples were incubated for 3 d in an orbital shaker at 150 rpm and 25 °C. Samples were then centrifuged (5804R centrifuge, Eppendorf International, Hamburg, Germany) at 11 000 rpm for 2 min at 26 °C, and 4 mL supernatant was removed and transferred to new tubes. To quantify the solubilized P-PO_4, for method of Sadzawka et al. (2004), and Undurraga et al. (2017) was applied with modifications. On 0.5 mL of supernatant, 1.5 mL distilled water were added plus 8 mL of the color development reagent.

IAA production of PGPR selected

Production of indole acetic acid (IAA) was evaluated for the 12 selected bacterial strains. A 100 µL cell suspension was inoculated into 30 mL capacity glass test tubes with 5 mL nutrient broth (Merck), which were shaken for 24 h at 150 rpm at 25 °C. From the bacterial suspension, 1 mL was transferred into 2 mL eppendorf tubes, which were centrifuged at 10000 rpm for 10 min, then 600 µL supernatant were transferred to an eppendorf tube and 400 µL modified Salkowski reagent (0.5 M $FeCl_3$ and 35% perchloric acid) were added. After 30 min of standing at room temperature, absorbance was measured in a spectrophotometer (MRC, POP OPTIZEN BIO, Korea) at a wavelength of 535 nm. The calibration curve was determined using IAA at concentrations from 0 to 50 mg L^{-1}. The absorbance data and IAA concentrations were adjusted to a linear regression ($R = 0.999$; $P = <0.001$).

Evaluation of nodulation rate in alfalfa plants

The strains GN-2, selected for higher IAA production and phosphate solubilization, GN-4 selected for phosphate solubilization, and GN-8 for ACC deaminase activity, were co-inoculated with *E. meliloti* (AG-06) on alfalfa seedlings. Incu tissue vials 500 cm^3, were filled to half volume with a sterilized substrate consisting of rock mineral wool and vermiculite 1:1 (v/v). Alfalfa seeds 'Alta Sierra Illapata' were disinfected by immersion in separate solutions of 70% (v/v) ethanol for 1 min, 3% (v/v) sodium hypochlorite 3 min, and then washed six times in sterile distilled water. Six seeds were sown per vial, and after emergence they were thinned to three. Once emerged, seedlings were inoculated with 1 mL bacterial suspension in 1% (w/v) sucrose at a DO_{600} nm of 0.1, additionally 20 mL N-free nutrient solution was added (0.31 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.21 g L^{-1} KH_2PO_4; 0.44 g L^{-1} K_2SO_4; 0.06 g L^{-1} FeEDTA; 0.05 g L^{-1} $CaSO_4$; 0.116 mg L^{-1} H_3BO_3 0.116; 0.0045 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$; 0.134 mg L^{-1} $ZnSO_4 \cdot 7H_2O$; 0.01 mg L^{-1} $MnSO_4$, $4H_2O$ and 0.06 mg L^{-1} $CoSO_4 \cdot 7H_2O$). An uninoculated control was also included. The pots were kept in a phytotron with high pressure sodium lamps (400 W Gro-lux, Osram Sylvania Ltd., Danvers, Massachusetts, USA) with a minimum photosynthetic photon flux density (PPFD) of 400-500 mol m^{-2} s^{-1}. Nodulation was assessed at 15, 25, and 35 d after inoculation. Four replicates were used per treatment applied.

Endophytic capacity of bacterial strains

Using the plants from the previous assay the endophytic capacity of the bacterial strains was determined according to the protocol by Parsa et al. (2013). Root and shoot fragments 3 cm long were washed in distilled water and disinfected superficially in 70% ethanol for 2 min, followed by immersion in 1% hypochlorite for 30 s, and finally four washes in sterile distilled water. The fragments were cut into 1 cm segments and sowed in standard nutrient agar plates, incubated for 48 h at 25 °C. 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. The same procedure was also performed with the nodules.

The obtained isolates were characterized by BOX-PCR genomic fingerprint using the primer BOXA1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Guiñazú et al., 2013) in order to verify if they corresponded to the same strain applied in the inoculation treatments. The cell template was prepared by suspending three loops full of bacterial cells in sterile saline solution, which were washed 3 times via centrifugation at 5000 g for 5 min, and suspension in nuclease-free water, adjusted to OD_{600} = 1.5. The sample was then subjected to a heat shock of 65 °C for 5 min and -20 °C for 5 min. The BOX-PCR reaction mixture consisted of 5.0 µL cell suspension, 0.3 µL GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA), 5.0 µL primer BOXA1R (10 µM), 1.5 µL $MgCl_2$ (25 mM), 5.0 µL 5x GoTaq Buffer (Promega), 0.5 µL dNTPs (10 mM) and 7.7 µL ultra pure water (Fisher Biotech, Wembley, Western Australia, Australia) to complete 25 µL (Marques et al., 2008). The PCR conditions were 95 °C for 7 min, 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 8 min, with a final extension at 72 °C for 16 min (Guiñazú et al., 2013). PCR products were analyzed by pre-stained 2% (w/v) agarose gel electrophoresis with 1:10 000 GelRed DNA gel stain (Biotium, Fremont, California, USA). Electrophoresis was carried out in the 1 × TAE buffered electrophoresis chamber (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 3 h, then the bands were visualized in a UV transilluminator.

Identification of bacterial isolates

To identify the bacterial strains selected for the plant assay, partial amplification and sequencing of 16S ribosomal DNA gene was performed, using the primers 8 F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGCTACCTTGTTACGACTT) (Sepúlveda-Caamaño et al., 2018). A reaction mixture was prepared with 2 µL cell preparation, 0.5 µL Taq polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), 1 µL primers 8F and 1492R, 6.0 µL 25 mM $MgCl_2$, 20.0 µL 5x Fisher-Biotech polymerization Buffer and 69.5 µL ultra pure water to complete 100 µL per reaction. The PCR cycles were: 7 min at 95 °C followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 45 s, finally 72 °C for 5 min. Amplification of the genes was verified by electrophoresis. The PCR products were purified and sequenced at Macrogen Inc. (Seoul, Korea). Partial sequences of the 16S rRNA genes were analyzed with GeneTool Lite 1.0 software (2000, Doubletwist, Inc., Oakland, California, USA). Sequence alignment and phylogenetic analyzes were carried out in MEGA 5.2 (Tamura et al., 2011). The phylogenetic tree was constructed using the Neighbor-joining test with 1000 replicates. Type strains included in the phylogenetic tree were obtained from GenBank of the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA).

Greenhouse experiment

The experiment was carried out in a polycarbonate greenhouse at the Instituto de Investigaciones Agropecuarias (INIA), Quilamapu facilities, Chillán, Chile. Pots consisted in PVC plastic tubes of 11 cm in diameter × 100 cm high (11 L volume). The substrate was a mixture of perlite and vermiculite 1:1 (v/v), with a water retention capacity on a dry weight basis of 172%. The substrate was autoclaved at 90 °C for 15 min. Alfalfa seeds were disinfected as described before. Six seeds were sown in each pot, and 10 d after sowing were thinned to two. Plants were irrigated with 20 mL of the N free nutrient solution at a weekly basis.

The strains used in this trial were GN-8 isolated from alfalfa and AG-30 (YsS6 WT) as a positive control (Rashid et al., 2012), both with ACC deaminase activity, in co-inoculation with *E. meliloti* AG-06. The inoculation was carried out at plant emergence with 1 mL bacterial suspension at a DO_{600} of 0.06. In addition, 1 mL *E. meliloti* AG-06 inoculum at DO_{600} of 0.1 was added. The inoculation was repeated after 8 d. Nodulation, root development, and aerial dry weight evaluations were performed 60 d after sowing. The root was carefully harvested, cleaned and washed free of soil. The root length was measured and the number of nodules per plant was determined. The nodules were placed in Eppendorf tubes and dried for 48 h at 70 °C, to determine their dry weight. Data were analyzed through an ANOVA and the comparison of means through the LSD test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Characterization of bacterial strains

A total of 32 bacterial strains were isolated from alfalfa rhizosphere from Cauquenes, a dryland area marked by water deficit (del Pozo et al., 2017). Among plant growth promoting bacteria, there are those that live in the exorhizosphere of plants and others that are found in the tissues of roots and stems (Nascimento et al., 2012), showing different specific functions that promote plant growth and development, especially in soils with limited nutritional levels or with adverse physical and chemical properties for plant growth and development (Egamberdiyeva, 2007). Since the rhizobacteria isolates were intended to be used as co-inoculants to improve the symbiotic performance of *E. meliloti* strain AG-06, 20 of the 32 isolates were discarded as they were incompatible with AG-06 showing antagonistic activity towards the rhizobial inoculant. Thus, the remaining 12 strains are shown in Table 1. The selection of PGPR strains compatible with the specific rhizobia has enabled successful inoculation and improvements in many aspects of growth in some legume crops such as soybeans, beans and chickpeas (Masciarelli et al., 2014), nodulation rates being the most favored in both, optimal growth conditions and under abiotic stress (Yadav and Prakash, 2014).

The 12 selected strains produced IAA at varying concentrations, ranging from 13.71 to 26.22 mg IAA L^{-1} (Table 1). Among bacterial strains, GN-2, GN-7, GE-4 and GE-5 outstood for producing significantly higher amounts of IAA compared to other strains. IAA is a key regulator of plant growth, as it participates in metabolic processes such as elongation and cell division, apical dominance, tropism and vascular differentiation (Glick, 2014). The synthesis of bacterial IAA is generated from tryptophan as a direct precursor through three synthesis pathways (Duca et al., 2014), and its concentration is regulated by the ability of each bacterial strain and not by the concentration of tryptophan (Rashid et al., 2012). The effect of bacterial IAA production on plants has been widely reported in different crops (Ali et al., 2004), significantly improving physiological and agronomic parameters (Barrucha et al., 2013). IAA compounds play an important role in the formation of lateral and adventitious roots (Kisiel and Kepczynska, 2016), and in the proliferation of cortical cells at the time of nodulation and expression of ligated genes in early nodulation (Suzaki et al., 2013).

Of the 12 bacterial strains evaluated for phosphate solubilization, eight formed solubilization halos on Pikovskaya agar. The eight strains showed solubilization rates between 14.7 and 93.8 mg P-PO_4 L^{-1} solubilization according to the P-Olsen test, with GN-7 and GN-9 strains being superior, although statistically similar to GN-2 and GN-11 (Table 1). Phosphorus is the second most important nutrient for crops, but many soils worldwide are deficient in this mineral (Oufdou et al., 2016). The importance of phosphate-solubilizing bacteria in agriculture is well grounded as biofertilizers (Sharma et al., 2013; Oufdou et al., 2016), because of the ability of bacteria to solubilize the inorganic P of the soil through diverse processes into phosphate that is available to plants, increasing crop production (Chaiharn and Lumyong, 2011).

Table 1. Bacterial strains isolated from rhizosphere of alfalfa: compatibility with *Ensifer meliloti*, indol acetic acid (IAA) production, phosphate solubilization and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity.

Strain	Origin	Phosphate solubilization PO_2	IAA production	ACC deaminase activity
		mg L^{-1}		
GN-1	Endorhizosphere	14.70d	18.14bc	-
GN-2	Endorhizosphere	90.73ab	26.22a	-
GN-4	Endorhizosphere	15.13c	15.13c	-
GN-7	Endorhizosphere	93.77a	22.47ab	-
GN-8	Endorhizosphere	-	14.03c	+
GN-9	Endorhizosphere	93.37a	19.07bc	-
GN-15	Endorhizosphere	-	15.70bc	-
GN-18	Endorhizosphere	-	13.71c	-
GE-4	Exorhizosphere	80.50b	19.70abc	-
GE-5	Exorhizosphere	-	20.13abc	-
GE-6	Exorhizosphere	88.40ab	16.79bc	-
GE-11	Exorhizosphere	87.05ab	17.18bc	-

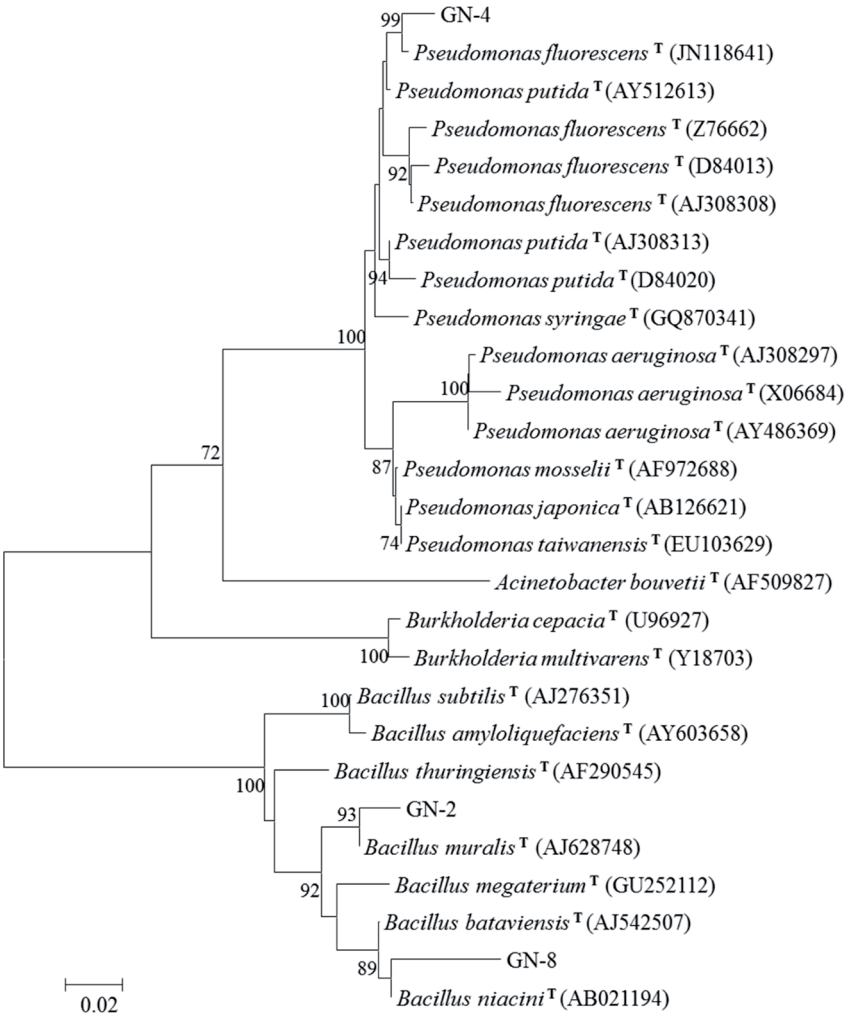
Different letters represent significant differences according to LSD test ($P < 0.05$).
GN: Isolated from the endorhizosphere; GE: isolated from the exorhizosphere.

Of the evaluated strains only the bacterial strain GN-8 showed ACC deaminase activity (Table 1). The enzyme ACC deaminase degrades and consumes the precursor of ethylene 1-amino-cyclopropane carboxylic acid (ACC) (Glick, 2014), or inhibits the enzyme ACC synthase (Hardoim et al., 2008). Upon breaking the ethylene precursor ACC, it is converted to alpha-ketobutyrate and ammonium, providing the bacteria with a source of N (Penrose and Glick, 2003). The ACC deaminase activity has been reported to improve nodulation in legumes by counteracting inhibition in the elongation of infection threads and decreasing the senescence rate of nodules caused by ethylene (Hardoim et al., 2008; Zafar et al., 2012; Glick, 2014).

Identification of PGPR strains

Bacterial strains GN-2, GN-4, and GN-8 were identified at the species level by amplification and sequencing of the partial 16S rRNA gene. The amplified fragment was 1200 bp. The phylogenetic tree of the 16S rRNA shows that the strain GN-4 is grouped in the *Pseudomonas* clade, with a sequence similarity of 99% to *Pseudomonas fluorescens* (Figure 1). However, the GN-2 and GN-8 strains were grouped in the *Bacillus* clade, specifically GN-2 was close to *Bacillus muralis* with 99% sequence similarity and GN-8 to *Bacillus niacini* (Figure 1). Bacterial strains of the genus *Pseudomonas* and *Bacillus* have been reported as plant growth promoters in alfalfa (Guiñazú et al., 2013; Kisiel and Kepczynska, 2016), with ability to synthesize IAA, to solubilize P and showing ACC deaminase activity (Rashid et al., 2012; Kisiel and

Figure 1. Neighbor joining phylogenetic tree based on 16S rRNA sequencing of bacterial isolates GN-2, GN-4, and GN-8. 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).



Kepczynska, 2016). Since the sequencing of the 16S rRNA gene is not always sufficient to determine a species, because it is a slowly evolving gene compared to other housekeeping genes (Sepúlveda-Caamaño et al., 2018), sequencing of other conserved genes will be necessary to obtain the definitive identification.

Endophytic capacity

From the plant tissue samples of alfalfa plants inoculated with PGPR under controlled conditions, 44 bacterial isolates were obtained. However, the BOX-PCR fingerprints showed that only the strain GN-4 could be recovered from plant tissues being able to colonize upper tissues such as stem. Bacterial endophytes can enter seeds through vascular connections, or directly through gametes colonizing the embryo (Truyens et al., 2015). All strains used for the plant trial were initially isolated from the alfalfa endorhizosphere. However, according to these results, the colonization of the endorhizosphere does not necessarily ensure its establishment as an endophyte in other tissues of the plant. The endophytic capacity of a bacterium is based on genomic differences in relation to soil rhizospheric bacteria (Ali et al., 2014), and also on the production of metabolic compounds necessary to overcome the microbiological competition in the rhizosphere, which endophytes need to colonize before invading plant tissue (Brader et al., 2014).

Effect of co-inoculation *E. meliloti* + PGPR on early nodulation

All the alfalfa plants were nodulated 15 d after inoculation, but plants inoculated with PGPR plus *E. meliloti* (AG-06) outstood in nodulation compared to treatment with only AG-06 although nonsignificant differences were found at this point (Table 2). Twenty-five days after inoculation the nodulation was significantly higher ($P < 0.05$) when co-inoculated with GN-8 (ACC deaminase producer) compared to treatments co-inoculated with the other PGPR and to the control treatment inoculated only with *E. meliloti* (AG-06) (Table 2). After 35 d, nodulation was similar for all inoculation treatments (Table 2). In legumes, where N nutrition depends on the biological fixation of atmospheric N, the number of nodules in the roots is indispensable for this process (Masciarelli et al., 2014). The strain GN-8 with ACC deaminase activity had a greater effect on nodulation compared to the IAA-producing strain GN-2 (Table 2). The presence of ACC-deaminase improves auxin response factor synthesis and reduces ethylene levels in the nodules (Ghosh et al., 2015). This allows the accumulation of IAA favoring the formation of nodules and other mechanisms of growth promotion (Ghosh et al., 2015). Also the reduction of ethylene levels by ACC-deaminase, promotes the elongation of infection thread in nodule formation and decreases the senescence rate of nodules (Glick, 2014). In this trial, *Sinorhizobium*-PGPR co-inoculation had no effect on root growth on alfalfa (Table 2).

Greenhouse trial

The effect of the bacterial strain with ACC deaminase activity GN-8 on nodulation on alfalfa was evaluated under greenhouse conditions and compared to the effect of AG-30 (YsS6 WT) (Rashid et al., 2012). The nodulation was higher in both inoculated treatments, resulting in an increase in the number of nodules of 18% with GN-8 and 118% with AG-30 in relation to the control inoculated with *E. meliloti* only. However, this improvement was significant only in the case of AG-30 ($P < 0.05$) (Table 3). Exogenous expression of an ACC deaminase gene in *Mesorhizobium ciceri* has increased nodulation in chickpea significantly up to 200% (Nascimento et al., 2012). A study by Guiñazú et al. (2013) shows similar results of the effect of bacterial strains of the genus *Bacillus* and *Pseudomonas* on nodulation in

Table 2. Effect of inoculation the *Ensifer meliloti* AG-06 and plant growth promoting rhizobacteria (PGPR) on nodule score and root length in alfalfa in phytotron chambers.

	Root length		Nodule score	
Inoculants	cm	15 d	25 d	35 d
AG-06	3.60ns	1.5ns	5.50c	10.5ns
AG-06+GN-2	4.55	3.0	13.25b	13.0
AG-06+GN-4	4.30	2.5	5.25c	10.5
AG-06+GN-8	4.23	4.0	15.50a	13.5

Different letters in column represent significant differences according to LSD test ($P < 0.05$).
ns: Nonsignificant.

Table 3. Effect of inoculation of *Ensifer meliloti* and plant growth promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity in alfalfa nodule number, nodule dry weight, root length and crown diameter under greenhouse conditions.

Inoculants	Nodule number	Nodule dry weight	Root length	Crown diameter
		mg	cm	mm
AG-06	11b	0.0033^{ns}	30.23^{ns}	1.63^{ns}
AG-06+GN8	13b	0.005	36.83	1.96
AG-06+AG-30	24a	0.0052	27.6	1.91
CV	25.55	43.85	15.14	25.65

Different letters represent significant differences according to LSD test ($P < 0.05$).
ns: Nonsignificant; CV: coefficient of variation.

alfalfa. The use of endophytic bacteria with enzymatic activity, in addition to rhizobia with genetic expressions of ACC deaminase, rapidly reduce ethylene levels during the nodulation process (Sarma and Saikia, 2014; Kisiel and Kepczynska, 2016). Ethylene in plants reacts rapidly to environmental and biological stress conditions through transcription of genes encoding a defense response in plants (Glick, 2014). In legume nodulation ethylene blocks the initiation of the infection thread and modulation of the Nod factor, especially under conditions of environmental stress where the concentration of ethylene is induced at levels unfavorable for the development of the plants (Glick, 2014). The dry weight of the nodules and the diameter of the crown obtained for the treatments *E. meliloti*-PGPR was higher than the treatment inoculated only with *E. meliloti* (Table 3). There were no differences between treatments in root length and in the crown diameter (Table 3). In this experiment, plants were regularly watered and fertilized and were under controlled climatic conditions. Yet, the activity of ACC deaminase strains is known to increase when plants are exposed to stress (Sarma and Saikia, 2014) due to the increase of ethylene, significantly improving growth and symbiotic efficiency during nodulation (Kisiel and Kepczynska, 2016). Therefore, improved plant growth promoting results could be expected from these strains under field or stressful conditions.

CONCLUSIONS

Plant growth promoting bacterial strains were isolated from the alfalfa rhizosphere in a Chilean dryland soil, showing different growth promoting properties such as indole acetic acid production, phosphate solubilization and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. The ACC deaminase activity of bacteria associated with the rhizosphere significantly improves early nodulation in alfalfa plants under greenhouse conditions.

ACKNOWLEDGEMENTS

This study was carried out thanks by the financial support Project FONDECYT Nº 11130479. The authors thank Dr. Bernard Glick, for kindly providing strains with ACC-deaminase activity and mutants.

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