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Rizobacterias asociadas a lenteja (*Lens culinaris* L.) y su efecto en la nodulación en coinoculación con rizobio

Tesis para optar al grado de Magíster en Ciencias Agronómicas con mención en Producción y Protección Vegetal

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RIZOBACTERIAS ASOCIADAS A LENTEJA (*LENS CULINARIS* L.) Y SU EFECTO EN LA NODULACIÓN EN COINOCULACIÓN CON RIZOBIO

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Lentil (*Lens culinaris* L.) growth promoting rhizobacteria and their effect on nodulation in coinoculation with rhizobia

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INTRODUCCIÓN GENERAL

Dentro de las leguminosas de interés para la alimentación humana se encuentran *Phaseolus vulgaris* L., *Vicia faba* L., *Pisum sativum* L., *Lens culinaris* M., *Cicer arietinum* L., *Vigna radiata* L. y *Glycine max* L. Entre estas especies, la lenteja (*Lens culinaris* M.) es rica en proteína, fibra, vitaminas y minerales, ubicándose en sexto lugar del ranking global de producción de granos secos, con una produción de 3.787.000 t (2004 - 2006), siendo los principales productores Canadá, India y Turquía (Erskine *et al*., 2009). En Chile, la lenteja se cultiva normalmente en la zona del secano por tener una menor incidencia de enfermedades fungosas y por ser un cultivo con bajos requerimientos hídricos (Peñaloza *et al.*, 2007; Tay *et al.*, 2001). Debido a que gran parte de los suelos del secano se encuentran erosionados y son de baja fertilidad, además de la escasa incorporación de tecnología por parte de los agricultores de esa zona para aumentar su competitividad, no se logra obtener rendimientos capaces de competir con productores internacionales (Peñaloza *et al.*, 2007; Tay *et al.*, 2001). Banfi (2013) señala que desde el año 2.000 la superficie de lentejas en Chile se encuentran prácticamente estancadas, sembrandose entre 1.000 y 1.200 hectáreas aproximadamente, con un rendimiento promedio de 7 qq ha^{-1}, generando una producción no mayor de 800 a 1.000 toneladas. De acuerdo a ODEPA, el año 2013 se importaron 7.897 ton de lentejas, donde el consumo doméstico estaría cubierto en más del 90% por lentejas importadas principalmente de Canadá (Banfi, 2013).

Las leguminosas agrupan distintas especies de plantas que fijan nitrógeno atmosférico y mantienen la fertilidad de los suelos, debido a la asociación simbiótica existente con bacterias de los géneros *Rhizobium, Bradyrhizobium, Mesorhizobium* y *Sinorhizobium,* entre otros, conocidas comúnmente como rizobios (Zahran, 2001). Factores ambientales como la sequía afectan el establecimiento de la relación simbiótica, donde la baja humedad y la alta temperatura del suelo producen daños a la planta, además de disminuir el porcentaje de sobrevivencia de la bacteria (Nascimento *et al*., 2012a).

El establecimiento de la relación rizobio-leguminosa, es un proceso que involucra una serie de etapas y eventos coordinados que van a definir la especificidad de la relación y también la efectividad. Finalizado el proceso, el rizobio induce la formación de nódulos en las raíces de las plantas, donde se realiza la fijación de nitrógeno (Masson-Boivin *et al*. 2009; Oldroyd y Downie, 2008; Gibson *et al*., 2008; Taiz y Zeiger, 2006). El establecimiento de esta simbiosis involucra la acción de genes específicos (*nod)* presentes

en las bacterias y el intercambio de señales químicas entre las plantas hospederas y los rizobios (Oldroyd y Downie, 2008). A través de las raíces, las plantas de leguminosas secretan atrayentes químicos ((iso) flavonoides y betaínas) los que provocan una migración de los rizobios a las paredes celulares de los pelos radicales. Durante la formación del nódulo radical ocurre simultáneamente la colonización y la organogénesis del nódulo (Taiz y Zeiger, 2006). Por su parte, los rizobios sintetizan oligosacáridos, los cuales inducen una curvatura pronunciada de las células de los pelos radicales, donde los rizobios quedan atrapados en pequeños compartimientos, los que degradan la pared celular ingresando directamente al exterior de la membrana plasmática (Oldroyd y Downie, 2008). Posteriormente se produce una fusión de vesículas derivadas del aparato de Golgi causando una extensión tubular de la membrana plasmática, formando un 'tubo de infección'. Las células corticales forman un área diferenciada llamada 'primordio del nódulo', a partir del cual se desarrolla el nódulo (Oldroyd y Downie, 2008). El 'tubo de infección', saturado con rizobios, crece a lo largo de los pelos radicales y de las capas de la células corticales, hasta llegar a las células especializadas del nódulo (Gibson *et al*., 2008), fusionándose el 'tubo de infección' con la membrana plasmática del hospedero, liberándose las bacterias en el apoplasto, donde ya dentro del nódulo, las bacterias infectan a gran cantidad de células (Gibson *et al*., 2008). Posteriormente, se desarrolla el sistema vascular en el nódulo para intercambiar el nitrógeno fijado por el rizobio, mientras que la planta hospedera le suministra otros nutrientes y carbohidratos (Taiz y Zeiger, 2006). La fijación biológica de nitrógeno convierte el nitrógeno atmosférico (N_2) en amoniaco (NH_3), el cual se ioniza a amonio (NH_4), y es transformado rápidamente en formas orgánicas, reacción catalizada por el complejo enzimático nitrogenasa en condiciones de anaerobiosis (Gibson *et al*., 2008). Las leguminosas transportan el nitrógeno orgánico desde los nódulos al tallo, a través del xilema, exportándolo como amidas o ureidos (Taiz y Zeiger, 2006).

En países como Australia, Pakistán (Athar, 1998), Portugal y Canadá (Nascimento *et al*., 2012b) se ha estudiado la relación leguminosa-rizobio (*Rhizobium spp.*, *Mesorhizobium spp.*) y el efecto que distintos niveles de estrés ambiental pueden provocar sobre el desempeño simbiótico. Athar (1998) probó la tolerancia a la sequía con distintas cepas de rizobios, aislados de diversos sectores de Pakistán, obteniendo una mayor sobrevivencia de aquellos aislados provenientes desde zonas con mayor sequía y salinidad. Lo mismo, señala Zahran (2001) para condiciones áridas y semi-áridas. También Howieson y Ballard (2004)

reportan pérdida de viabilidad de rizobios y disminución en la nodulación y fijación de nitrógeno ante condiciones de estrés edáfico, las cuales pueden ser subsanadas con la selección del rizobio apropiado para tales condiciones y a través de manejo agrícola.

Por otro lado, se ha investigado la posibilidad de incrementar la tolerancia a estrés hídrico de leguminosas y rizobios por medio de la co-inoculación con bacterias simbióticas residentes en la rizósfera, las cuales pueden promover el crecimiento radicular y producción, siendo en esos casos llamadas PGPR (del inglés *plant growth-promoting rhizobacteria*) (Sarma y Saikia, 2014; Rashid *et al.*, 2012).

Las PGPR promueven el crecimiento a través de diversos mecanismos, tales como: la solubilización de fósforo, la fijación de nitrógeno y la producción de fitohormonas (Rashid *et al.*, 2012).

El ácido indol acético (AIA) es una hormona sintetizada por plantas y por bacterias que posee la capacidad de controlar el crecimiento y desarrollo de la planta y también de algunas bacterias, ya que actúa como molécula señal para el control de la expresión de diversos genes (Duca *et al*., 2014). El AIA bacteriano puede tener efectos benéficos y deletéreos en la planta hospedera, lo cual dependerá principalmente de la concentración de AIA sintetizado y la sensibilidad de la planta hospedera. La bacteria puede estimular el crecimiento tanto por la síntesis de AIA o regulando las concentraciones de esta hormona (Duca *et al*., 2014).

Además del mejoramiento genético de cultivos y la transgenia, la aplicación de bacterias promotoras de crecimiento es considerada como promisoria para mejorar la tolerancia a estrés abiótico en los cultivos (Sarma y Saikia, 2014). Una de las estrategias de las PGPR para inducir la tolerancia a estrés en plantas es la producción de 1-aminociclopropano-1-carboxilato (ACC) deaminasa. De acuerdo a Penrose y Glick (2003), existen bacterias rizoféricas con un amplio rango de actividad ACC deaminasa. En tejidos vegetales, el ACC es un precursor del etileno y es sintetizado por la acción de la enzima ACC sintetasa sobre S-adenosilmetionina. Cantidades pequeñas de etileno inciden en el crecimiento vegetal ya que es la hormona relacionada con la senescencia y abscisión de las hojas, y la maduración de frutos. Éste aumenta su concentración en la planta, en respuesta a condiciones de estrés y/o al tener daños físicos, químicos o mecánicos en los órganos vegetales (Pérez y Martínez-Laborde, 1994), lo cual puede causar el inicio de la senescencia, clorosis y finalmente la muerte de la planta. Gage (2004) y Nascimento *et al*.

(2012b) señalan que el etileno inhibe la formación y desarrollo de nódulos en varias leguminosas. Algunas PGPR de vida libre poseen la habilidad de sintetizar la enzima ACC deaminasa promoviendo el crecimiento de las plantas en condiciones de estrés, tales como: sequías, inundaciones y ataque de patógenos. La función de esta enzima, es secuestrar y romper el ACC, y de esta manera prevenir que concentraciones nocivas de etileno sean acumulados en los tejidos de la planta (Ali *et al.*, 2012), reduciendo su concentración y sus efectos deletéreos en el desarrollo y crecimiento de la planta (Glick *et al.*, 2007). Nascimento *et al*. (2012b) señalan que algunas cepas de *Mesorhizobium* sintetizan ACC deaminasa, lo cual se ha traducido en la promoción de la formación del nódulo. Otros investigadores han reportado que existen bacterias del género *Pseudomonas* con la habilidad de sintetizar la enzima ACC deaminasa, la cual sería la responsable del retraso de la senescencia en flores (Ali *et al.*, 2012). Sarma y Saikia (2014) inocularon *Pseudomonas aeruginosa* al cultivo *Vigna radiata* L. observando una mayor tolerancia al estrés hídrico. Las plantas inoculadas produjeron mayor biomasa, tuvieron un incremento del crecimiento y desarrollo en condiciones de estrés en comparación con el control. De acuerdo a Penrose y Glick (2003) y a Chang *et al.* (2014), las bacterias deben tener algún grado de estrés para actuar como promotoras. La tolerancia a estrés hídrico estaría asociada a las habilidades promotoras de producción de sideróforos, HCN (Cianuro de hidrógeno), AIA, ACCdeaminasa y solubilización de fósforo y a una acumulación significativamente mayor de enzimas antioxidantes y de osmolitos celulares, sumado al incremento en la activación de genes de respuesta al estrés hídrico en plantas inoculadas bajo condiciones de sequía (Sarma y Saikia, 2014).

Algunos investigadores señalan que las PGPR complementadas con rizobios mejoran la nodulación en leguminosas, por ende la fijación de nitrógeno, haciendo a estos (rizobios) más efectivos en condiciones de estrés (Sarma y Saikia, 2014; Nascimento *et al*., 2012a; Fox *et al*., 2011; Glick *et al.*, 2007). Nascimento *et al*. (2012a), señala que cepas de *Mesorhizobium* al producir la enzima ACC deaminasa, aumentan la nodulación en raíces de garbanzo (*Cicer arietinum* L.), además de promover el crecimiento de la planta y actuar como biocontroladores.

La inoculación de un rizobio apropiado es recomendada como práctica de cultivo en leguminosas para tener buenos rendimientos. Se ha observado que en algunos casos, al inocular leguminosas, los rizobios seleccionados tienen una baja ocupación nodular y las

plantas tienden a nodular con poblaciones de rizobios nativos, los cuales no son necesariamente efectivos. Las PGPR pueden ayudar al rizobio inoculado y a la sobrevivencia sinérgica incrementando la nodulación inicial y la fijación de nitrógeno (Zafar *et al*., 2012; Kumar y Chandra, 2008).

En Chile se han realizado estudios de inoculación con rizobios en leguminosas (Baginsky *et al.,* 2015; Ovalle *et al*., 2015). Sin embargo, no existen estudios del sinergismo entre rizobios y PGPR nativos, desconociendo el efecto que la co-inoculación pueda tener sobre la nodulación, parámetros productivos y la tolerancia a estrés en lentejas.

HIPÓTESIS

La co-inoculación de PGPR y rizobios en plantas de lenteja, anticipan e incrementan la nodulación y aumentan la producción de biomasa de las plantas.

OBJETIVO GENERAL

Evaluar el efecto en la promoción de crecimiento y nodulación con la co-inoculación de rizobacterias promotoras de crecimiento y rizobios en plantas de lentejas.

OBJETIVOS ESPECÍFICOS

- Seleccionar bacterias con habilidad de producir ACC deaminasa y ácido indol acético asociadas a la rizósfera de plantas de lenteja en suelos de zonas de secano.
- Evaluar el sinergismo/antagonismo entre las bacterias promotoras y rizobios asociados a lenteja.
- Identificar molecularmente cepas bacterianas promotoras de crecimiento obtenidas de la rizósfera de lentejas.
- Evaluar y comparar la nodulación y producción de biomasa en lenteja al coinocular con bacterias rizosféricas y rizobio (*Rhizobium leguminoasrium* var. viciae).

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Capítulo 1

Lentil (*Lens culinaris* L.) growth promoting rhizobacteria and their effect on nodulation in coinoculation with rhizobia

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ABSTRACT

Lentil is cultivated in Chilean Mediterranean drylands, in areas with soils that are nutrient depleted and eroded. Inoculation of lentil with rhizobia in co-inoculation with growth promoting rhizobacteria would allow higher biomass and an opportunity for early nodulation and increased nitrogen fixation. The objective of this research was to select rhizosferic bacteria (PGPR) from lentils and to evaluate their effect on lentil nodulation in co-inoculation with rhizobia. Sixty eight lentil rhizobacteria isolates where obtained from nine soils in the mediterrean area. These were fingerprinted through BOX1-PCR reducing the number to 62 distinct strains. The strains were evaluated for ACCdeaminase activity, IAA production and compatibility with rhizobia. Seventeen strains showed ACC-deaminase activity, all of them synthesized IAA and 38 were compatible with the rhizobia. Ten selected strains where identified as *Pseudomonas* spp. through 16S rRNA sequencing. The strains were inoculated in lentil seedlings growing on seed germination pouches, to evaluate nodule formation. The strain LY50a promoted early nodulation in comparison to the control with rhizobium (AG-84). In conclusion, bacteria from the rhizosphere from Mediterranean soils of Chile can be used as nodulation promoters in lentils.

Keywords: PGPR; *Pseudomonas*; *Rhizobium leguminosarum* bv. *vicieae*; indole acetic acid; ACCdeaminase

Introduction

In Chile, lentils are normally cultivated in dryland areas because of its lower water requirement and lower incidence of fungal diseases under those environmental conditions (Peñaloza et al. 2007; Tay et al. 2001). However, under these conditions, lentils reach low yields because much of the soils in Chilean mediterranean dryland are eroded, nutrient depleted, and farmers have little access to technology (Peñaloza et al. 2007; Tay et al. 2001).

Legumes fix atmospheric nitrogen and maintain soil fertility due to their symbiotic association with bacteria commonly known as rhizobia (Zahran 2001). Environmental factors such as drought affect the establishment of the symbiotic relationship, where low humidity and high soil temperature cause damage to the plant, in addition to reducing the percentage of survival of the bacteria (Nascimento et al. 2012a).

Several studies show how biotic and abiotic stresses can adversely affect nodulation (Nascimento et al. 2012b; Howieson and Ballard 2004; Athar 1998). Abiotic stresses such as drought and salinity can be largely overcome by selecting rhizobia strains with tolerance to these adverse environmental conditions (Zahran 1999; Athar 1998). In the same way, the coinoculation of rhizobia with rhizospheric bacteria, which can promote root growth and yield, is an alternative to increase abiotic stress tolerance in legumes (Sarma and Saikia 2014; Rashid et al. 2012).

Plant growth-promoting rhizobacteria (PGPR) promote growth through various mechanisms, such as phosphate solubilization, nitrogen fixation and phytohormones production (Rashid et al. 2012). In fact, some bacteria synthesize the hormone indole acetic acid (IAA) which controls the growth and development of the plant, acting as a signal molecule to control the expression of several genes (Duca et al. 2014a). Another strategy to improve stress tolerance in plants is through the regulation of the ethylene in plant tissues, through the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase by PGPR. This enzyme sequesters and cleaves the ACC, and thereby prevent the accumulation of harmful concentrations of ethylene in plant tissues (Ali et al. 2012) and its deleterious effects on plant growth and development (Glick et al. 2007).

The inoculation of legumes with effective rhizobia is a recommended agronomic practice that can increase yields. However, under stressful environments, selected

inoculants are less competitive than soil naturalized rhizobia populations, reducing considerably nodule occupation by the selected strain (Gerding et al. 2014; Mishra et al. 2011). In this situation, coinoculation of rhizobia with PGPR have allowed to obtain higher rates of nodulation, increase rhizobia survival, and higher presence of the inoculant in the nodules, which has been associated with an increase in nitrogen fixation effectiveness (Sarma and Saikia 2014; Nascimento et al. 2012a; Fox et al. 2011; Glick et al. 2007).

Lentil is in general a low return crop which derives from its poor productivity (Mishra et al. 2011; Tay et al. 2001). Therefore, there is a need for development of effective inoculants to increase lentil performance through nitrogen fixation (Athar 1998) and stress tolerance induction particularly under harsh edaphic and climatic conditions (Sarma and Saikia 2014; Nascimento et al. 2012c; Mishra et al. 2011; Shaharoona et al. 2011).

Objectives of this research were to select lentil rhizosphere bacteria based on indole acetic acid production and ACC deaminase activity from Chilean Mediterranean dryland enviroments and to evaluate their effect in coinoculation with *Rhizobium leguminosarum* bv. *vicieae* on plant growth promotion and nodulation in lentils.

Materials and methods

Rhizobacteria isolation

Seven soil samples were collected from three Chilean mediterranean dryland areas: two samples from Quitripin, Quirihue (36° 22'1,24 " S 72° and 36° 22'1,24 30'17,87"O "S 72° 30'9,38"O); three samples from Rincomavida, Portezuelo (36° 29'55,39 "S 72° 21'23,18"O; 36° 29'40,07"S 72° 22'29,21"O and 36° 30'9,85 "S 72° 21'35,54"O) and two samples from Los Quillayes, Yumbel (36° 59'26,56 "S 72° and 36° 59'20,45 33'55,17"O"S 72° 34'8,19"O). Two samples of the experimental station El Nogal of the University of Concepción, Chillán (36° 35'45.01 "S 72° 4'49.05" W and 36° 35'45.06 "S 72° 4'47.96" O), which is an irrigated field, were also considered in this research.

Each of the samples was used to establish a trapping experiment under controlled glasshouse conditions (Howieson et al. 2016). Lentil seeds of the cultivar Araucana INIA were surface sterilized and seeded in the pots. After 60 days the plants were removed and bacteria were isolated from the endorhizosphere and exorizosphere.

Isolation of thermotolerant bacteria were conducted by the methodology described by Ferreira et al. (2011), while isolation of bacteria from the genus *Pseudomonas* was performed according to the protocol described by Vlassak et al. (1992). After 48 h isolated colonies were selected, and reisolated to obtain pure cultures. The isolates obtained, were cryopreserved at -80°C in nutrient broth and glycerol (Oskouei et al. 2010).

Molecular fingerprinting

The genetic diversity of the isolates was assessed at the strain level by molecular fingerprinting using the primer A1R (5'- CTA CAA CGG GCT GAC GGC GAC G-3') (Versalovic et al. 1994) according to the methodology used by Guiñazú et al. (2013).

The templates were prepared using whole cells that were suspended in sterile saline solution and were centrifuged (5000 g by 5 min) and resuspended in nuclease-free water 3 times, adjusting the optical density (OD) to 1.5 at 600 nm wavelenght. Subsequently, cells were exposed to a thermal shock of 65°C by 5 min and then -20°C during 5 min.

The PCR reaction mix contained 5.0 µL of cell templated, 0.3 µL of Gotaq polymerase (Promega) (5 U μL^{-1}), 5.0 µL A1R primer (10 µM) 0.5 µL dNTPs (10 mM), 5.0 µL of the Gotaq buffer (5x) solution, 1.5 µL of $MgCl_2$ (25 mM) and 7.7 µL of ultrapure water, making a total of 25 µL (Marques et al. 2008). 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 (Guiñazú et al. 2013) with amendments in the time of PCR runs.

PCR products were analyzed by electrophoresis in 2% (w / v) agarose gels with 2.5 mL^{-1} µL Gel Red (10000x in DMSO, Biotium) in a 1x TAE buffer solution (40mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50 V for 3 hours. Bands were visualized in a UV transilluminator. The banding patterns were analysed to scan for presence of PCR product of specific molecular sizes. A binary matrix was constructed with the scores 1 or 0 for the presence or absence of a band at each molecular size. The matrix was then analyzed with AFPL SURV - software version 1.0 (Vekemans et al. 2002) to calculate the genetic distance among isolates. The distance matrix was then subjected to UPGMA cluster analyses using the NEIGHBOR application from the PHYLIP software package. The cladograms were visualized in Mega 5.2 (Tamura et al. 2011), distinguishing individual strains for the following tests and discarding repeated copies.

Assesment of indole acetic acid production (IAA)

IAA production was assessed for each individual bacterial strains isolated from lentil and for strains AG-45, AG-46, AG-49, AG-50 that correspond to nodular endophytes of the genus *Labrys* and AG-40 of the genus *Pseudomonas*, isolated from native legumes of the genus *Adesmia* (Gerding et al. 2016).

To quantify IAA, a calibration curve was made with IAA concentration between 0 and 50 µg mL^{-1}. An aliquot of 0.6 mL of each dilution was mixed with 0.4 mL of reagent Salkowski (98 mL perchloric acid 35%, 2 mL 0.5 M $FeCl_3$) (Sarwar and Kremer 1995). After 30 min of incubation at ambient temperature the absorbance was measured at a wavelength of 535 nm in a spectrophotometer (MECASYS, POP Optizen Bio, Korea). Data of absorbance and AIA concentration were fit to a linear regression ($R = 0.999$; $P = <0.001$) to obtain a calibration curve.

To evaluate IAA production by each isolates, 100 µL of cell suspension of 1×10^7 CFU mL^{-1} was inoculated in 3 mL of standard nutrient broth (Merck) and incubated at 25 ± 2°C in an orbital shaker (MRC, Tou-50, China) at 150 rpm by 48 hours. One mL of the bacterial suspension was placed in Eppendorf tubes, and centrifuged at 5000 rpm for 3 min at 16°C. A sample of 0.6 mL of the supernatant was mixed with 0.4 mL of reagent Salkowski and analyzed with a spectrophotometer to determine IAA concentration as described above.

ACC deaminase activity

To assess ACC deaminase activity of the strains, 100 µL of cell suspension (10^7 CFU mL^{-1}) was inoculated in 3 mL of nutrient broth, and incubated for 24 hours at 25 ± 2°C in an orbital shaker at 150 rpm. After incubation 1 mL was centrifuged at 8000 g for 10 min, removing the supernatant, and performing two washes with 1 mL of Dworkin and Foster (DF) broth [K_2HPO_4 4 g; Na_2HPO_4 6 g; $MgSO_4.7H_2O$ 0.2g; glucose 2 g; gluconic acid 2 g; citric acid 2 g; 0.1 mL solution $FeSO_4.7H_2O$; trace elements (10 mg H_3BO_3; $MnSO_4.H_2O$ 11.19 mg; $ZnSO_4.7H_2O$ 124.6 mg; $CuSO_4$. $5H_2O$ 78.22 mg; MoO_3 10 mg; sterile distilled water 100 mL) in 1000 mL sterile distilled water]. Samples were incubated in 1 mL of the DF broth in an orbital shaker for 24 hours. After incubation bacterial suspension were centrifuged and 500 µL of the supernatant were removed to concentrate the cells. A 5 µL droplet from each of the strains was poured on DF agar, on DF agar with added ACC (1-aminocyclopropane-1-carboxylic acid) and DF agar with $(NH_4)_2SO_4$ as nitrogen source

(Penrose and Glick 2003). Two ACC deaminase PGPR strains 8R6 Wild Type (WT) and YsS6 WT (identified as *Pseudomonas* sp and *Pseudomonas fluorescens*) were used as positive controls, while the mutant strains 8R6 acds and YsS6 acds were used as negative controls (Rashid et al. 2012). Colony growth on each media was assessed after 5 days using the ImageJ software.

Compatibility rhizobia - PGPR

The compatibility between selected rhizobacteria strains and the rhizobia strain *R. leguminosarum* bv. *viciae* AG-84 was evaluated. The rhizobacteria were grown on nutrient agar during 48 h at 25°C. After colony growth, cells were suspended in NaCl (0.89% (w / v)), and adjusted to an $OD_{600\ nm}$ of 0.1. An aliquot of 100 µL of each strain were inoculated in 900 µL nutrient broth 50% (w / v) and incubated at 25°C on a rotary shaker for 48 h. Cell suspensions were centrifuged at 5000 rpm for 5 min, then 60 µL of the supernatant were removed and transferred to microplates, along with 30 µL of rhizobia ($OD_{600\ nm}$ 0.25) in yeast mannitol broth. Controls with only rhizobia were also included. The microplates were incubated in a rotary shaker at 150 rpm at 25 ± 2°C. Rhizobial growth was assessed 24, 48 and 72 hours after inoculation on a microplate spectrophotometer (BioTek Epoch, USA) by measuring absorbance at 600 nm.

Strains that were compatible with rizobia, that showed high ACC deaminase activity and different levels of IAA production, were selected for next assays.

16S rRNA gene sequencing

To identify the selected strains at the species level, the 16S rRNA gene was sequenced. Bacterial templates were prepared as for the BOX PCR but adjusting the concentration to an $OD_{600\ nm}$ of 1.5. The primers 8F (AGAGTTTGATCCTGGCTCAG) (Turner et al. 1999) and 1492R (GGCTACCTTGTTACGACTT) (Tanaka et al. 2008) were used to amplify a 1500 bp internal region of the 16S rRNA. The cycling conditions were: 5 min at 95°C, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 30°C for 45 s, and finally at 72°C for 7 min. The amplification of the gene was verified through electrophoresis. The PCR products were purified and sequenced in Macrogen Inc. (Seoul, Korea). The chromatograms obtained were analysed and edited in the GeneTool Lite 1.0 (2000) software. Sequences alignments and phylogenetic analyses were conducted in MEGA 5.2 (Tamura et al. 2011). The phylogenetic tree was inferred by the maximum parsimony

algorithm with a bootstrap analysis based on 1000 replicates. Type strains sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI).

Effect of PGPR on lentil germination

From the total isolates obtained ten PGPR strains were selected, which were individually grown on standard nutrient broth and were standardized to an $OD_{600\ nm}$ of 0.1 in sucrose (1% w/v). Lentil seeds of the cultivar Araucana INIA were disinfected in sodium hypochlorite 4% (w / v) for 3 minutes, then in 70% ethanol (v / v) for 1 min, followed by 6 rinses in sterile distilled water. Seeds were imbibed in each bacterial suspension and were placed on water agar, an uninoculated control was also included. After 10 days, the number of germinated seeds was evaluated to calculate the percentage of germination.

Effect of PGPR on root development and plant nodulation in lentil

Lentil seeds were disinfected following the protocol described above and were deposited in autoclaved seed germination pouches (CYG pouch ®). Five seeds were included per pouch plus 10 mL of nutrient solution without nitrogen (Yates et al. 2016). When cotyledons had emerged seedlings were thinned to three and inoculated with 1 mL of bacterial suspension per seedling (0.5 mL of PGPR strains and 0.5 ml of rhizobia, at an $OD_{600\ nm}$ of 0.1). Plants were watered with 5 mL of water every 4 days and fertilized with 10 mL of nutrient solution devoid of N once a week (Yates et al. 2016). Pouches were placed in a plastic sealed box to prevent airborne contamination and maintained 25 ± 3°C in a phytotron with high-pressure sodium steam lamps (400 W Gro-lux®, Osram Sylvania Ltd., Danvers, MA, USA) providing a minimum photosynthetic photon flux density (PPFD) of 400-500 mol $m^{-2}\ s^{-1}$.

Nodulation was registered every 3 days during four weeks. Plants were removed from the pouches after 4 weeks and the total number of nodules and plant dry weight were assessed. The nodulation data on time was plotted, adjusted to an exponential curve to subsequently integrate the equation and estimate the area under the nodulation progress curve (AUNPC).

The final nodulation was evaluated using the nodule scoring system proposed by the Centre for Rhizobium Studies (2012), where each nodule was scored according to its size (1: ≤1mm; 2: 1 to 2 mm; and 3: >3 mm) and position on the root system.

The dry weight of each experimental unit (3 whole plants) was determined. The plants were deposited in paper bags and dried at 60°C until to obtain a constant weight.

Data were analyzed by an analysis of variance (ANOVA) and the mean comparison test LSD ($P \leq 0.05$) was performed when appropriate, with the software Infostat (Balzarini et al. 2008).

Results and discussion

The isolation of rhizospheric bacteria from lentil growing in different soils resulted in 63 isolates. After analysis of the BOX1-PCR fingerprinting patterns, the cladogram showed a great diversity of strains even from samples from the same site and host (Figure 1). Fifty seven isolates that had unique banding patterns were considered as individual strains and were selected for further studies while six isolates were excluded from future experiments because they revealed identical fingerprints to other isolates with BOX1 PCR. To assess the potencial of this group of individual strains is important considering that previous works encourage the use of bacterial inoculants isolated from local soils (Zafar et al. 2012), mainly because of their adaptation to the natural environment (Mishra et al. 2011). Moreover, it should be considered that lentil is a legume of agricultural and social importance in developing countries, specifically in areas with low fertility and eroded soils exposed to frequent droughts (Erskine et al. 2011).

The 62 strains tested (57 lentil strains and 5 *Adesmia* strains) were able to synthesize IAA, with concentrations ranging between 0.111 and 16.05 μg IAA mL^{-1}. Strains: LP 15; LQ 20; VCa 30; LCa 33; LCa 35; LP 37; LP 38; LP 39; LY 50a; LP 55; LP 56; LP 58; LQ 62 and LY 64 outstood for their IAA production (Supplementary material). Bacterial IAA is multifunctional, as it has physiological effects on plants such as cell division and elongation, root initiation and senescence. It is also considered a communication signal between plant and microbe (Duca et al. 2014a; Duca et al. 2014b) and is thought to increase bacterial adaptation to stress conditions, improving survival and persistence in the environment (Bianco et al. 2006).

Of the 62 strains, 17 showed ACC deaminase activity: VCa 1; LP 13; LP 15; LQ 23; VCa 30; LCa 32; LP 38; LY 50a; LY 50b; LQ 60; LQ 61; LY 66; AG 45; AG 46; AG 49 and AG 50 (Supplementary material). ACC deaminase has been found in several strains of

rhizobia and PGPR (Nascimento et al. 2012b; Duan et al. 2009; Ma et al. 2003). It can improve plant tolerance to different biotic and abiotic stresses as it cleaves the plant ethylene precursor, ACC, into ammonia and α-ketobutyrate (Glick 2015; Nascimento et al. 2012a)

In terms of compatibility, 24 rhizosphere strains inhibited the development of the rhizobia strain AG-84 (*Rhizobium leguminosarum* bv. *vicieae*), since the optical density of AG-84 was significantly reduced when growing in presence of rhizobacteria supernatant. These strains were therefore discarded from future experiments. The strains selected for nodulation asssessments are indicated in Table 1. Strains LCa 33, LCa 35, LP 37 and LP 39 were selected due to their IAA synthesis (5.62 and 16.04 µg IAA mL^{-1}); LY 50a, LQ 60 and LQ 61 especifically due to their higher ACC deaminase activity; and VCa 30, LP 38 and LY 66 because they were able to produce IAA and showed ACC deaminase activity.

PGPR strain identification

An internal fragment of the 16S rRNA gene of 1500 bp was successfully amplified for the 10 selected strains. According to 16S rRNA phylogenetic tree the strains clustered within the *Pseudomonas* clade (Figure 2). Strains within this genus have been described as able to induce growth of different plant species under stress conditions through the synthesis of IAA (Duca et al. 2014b; Rashid et al. 2012), antibiotics, siderophores (Mishra et al. 2011), phosphate solubilization (Rashid et al., 2012) and through enzymes such as ACC deaminase (Ali et al. 2014; Ali et al. 2012; Rashid et al. 2012; Penrose and Glick 2003). *Pseudomonas* strains have also been utilized as co-inoculants with rhizobia to improve nodulation and nitrogen fixation (Zafar et al. 2012; Mishra et al. 2011; Kumar and Chandra 2008).

Within the genus *Pseudomonas*, strains were distributed in five major clades. Strains LQ 60 and LQ 61 shared identical 16S rRNA sequences (99.89%) and were closely related to *Pseudomonas marginalis*. LCa 33 shared identical 16S rRNA sequence to *P. rhodesiae* (98%); LY 50a was close to *P. umsongensis*; and LY 66 shared identical sequences with *P. kilonensis* (98.8%) and *P. jessenii* (99.9%). Strains VCa 30, LP 39, LP 37 and LCa 35 clustered together and were similar to *P. granadensis*. In general there was no precise identification of the strains at the species level. The partial 16S rRNA, although considered an important housekeeping gene in bacterial identification, has been shown to be a slowly

evolving gene in comparison to other housekeeping genes and therefore lacks the required level of resolution to distinguish similar species (Gerding et al. 2012, Chelo et al. 2007; Eardly et al. 2005). For accurate identification of the strains at the species level, sequencing of additional housekeeping genes will be required.

Effect of coinoculation rhizobia PGPR on nodulation

Lentil seeds germination was higher than 98% in every inoculation treatment and there was no effect of the PGPR strains neither on percentage nor on the germination rate. There are reports of seed germination inhibition by PGPR inoculation (Jahanian et al. 2012), which is can be caused by IAA production (Glick 2015; Taiz and Zeiger 2006).

In the germination pouches, nodules were first detected 13 days after inoculation for some of the inoculation treatments (Figure 3). There were significant differences in the timing of nodulation within different inoculation treatments. Strain LY50a significantly improved the progression of nodulation (assessed through AUNPC) ($P \leq 0.05$) in comparison to other PGPR co-inoculants and to the rhizobial strain AG-84 on its own, where AUNPC was increased in 85%. There were also significant differences in nodule scoring index, where strain LY 50a co-inoculated with rhizobia induced higher values than rhizobia (AG-84) alone ($P \leq 0.05$) (Table 2). No statistical differences were found ($P > 0.05$) in terms of total nodule numbers nor in plant dry weight between treatments.

The strain that showed a positive effect on nodulation (LY 50a) was isolated from a soil in Yumbel, an area characterized by thin soils with moderate to good drainage and slightly undulating topography with stressful soil conditions for both crops and soil biota (Stolpe 2006). The advantage of strains adapted to these conditions is that they could survive in these soils (Figueiredo et al. 2010) and would be able to easily adapt and colonize roots when inoculated into the plant rhizosphere as has been described by Chen et al. (2006).

The strain LY 50a showed ACC deaminase activity. When ACC deaminase is present in legumes rhizosphere, the negative effects of ethylene on the process of nodulation are reduced (Shaharoona et al. 2011; Ma et al. 2003, 2004). A reduction of the ethylene levels on the rhizosphere contribute to bacterial colonization of the root and to the entry of rhizobia into root cells (Nascimento et al. 2012c). In fact, mesorhizobia containing ACC deaminase have proved to increase nodulation, rhizobia competitiveness and total plant

biomass (Nascimento et al. 2012a; Conforte et al. 2010). This is explained by the reduction on ACC levels, since ACC, as a precursor of ethylene, regulates early stages of rhizobial infection by inhibiting the perception of Nod factor, thus reducing the number of nodules (Gage 2004; Oldroyd et al. 2001). ACC and ethylene can also negatively influence nodule size and their maturation (Shaharoona et al. 2011; Tamimi and Timko 2003). A reduction on ethylene levels reduces Ca^{2+} spiking frequency and stimulate intracellular root hair invasion by rhizobia (Capoen et al. 2009). Moreover, Nascimento et al. (2012c), reported greater nitrogenase activity, and therefore more effective nodules in terms of nitrogen fixation in plants inoculated with ACC deaminase strains.

The strains that produced higher levels of IAA, LCa 35 and LP 37, did not induce higher nodulation in lentil than when inoculated with rhizobia only. However, the overall effect of IAA producing strains on further plant growth promotion and yield is yet to be tested. It is noted that the strain LCa 33 had a negative effect on nodulation and on plant growth, as nodulation score and dry weight were significantly lower than other PGPR treatments (Table 2). PGPR strains that produce IAA can have negative effects on plant development, as high concentrations of IAA inhibit root development (Soeno et al. 2010).

Inoculation with PGPR could further improve rhizobia competitiveness in the field, as it would induce rapid root hair invasion (Zafar et al. 2012; Mishra et al. 2011; Shaharoona et al. 2011), higher nodule occupancy by the selected rhizobial strain (Kumar and Chandra 2008) therefore suppressing subsequent infections of other naturalized and less effective rhizobia (Sato et al. 2003).

Conclusions

Rhizopheric bacteria isolate from lentil growing in nutrient depleted soils from Chilean Mediterranean drylands are able to synthesize indol acetic acid and ACC deaminase, and strain LY50a promotes early nodulation in lentil. This work is a first step in improving lentil nodulation and production in Chilean Mediterranean drylands, as co-inoculation with *Pseudomonas* species. Future studies might reveal the extent of these findings.

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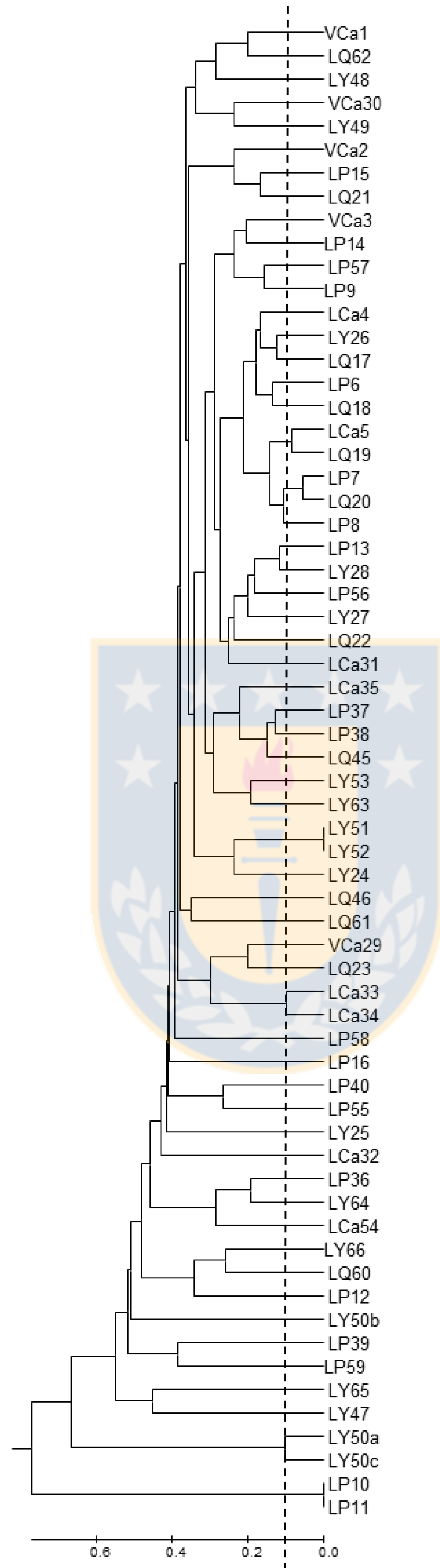

Figure 1. Cladogram generated through BOX1-PCR fingerprinting, comparing lentil rhizobacteria isolates.

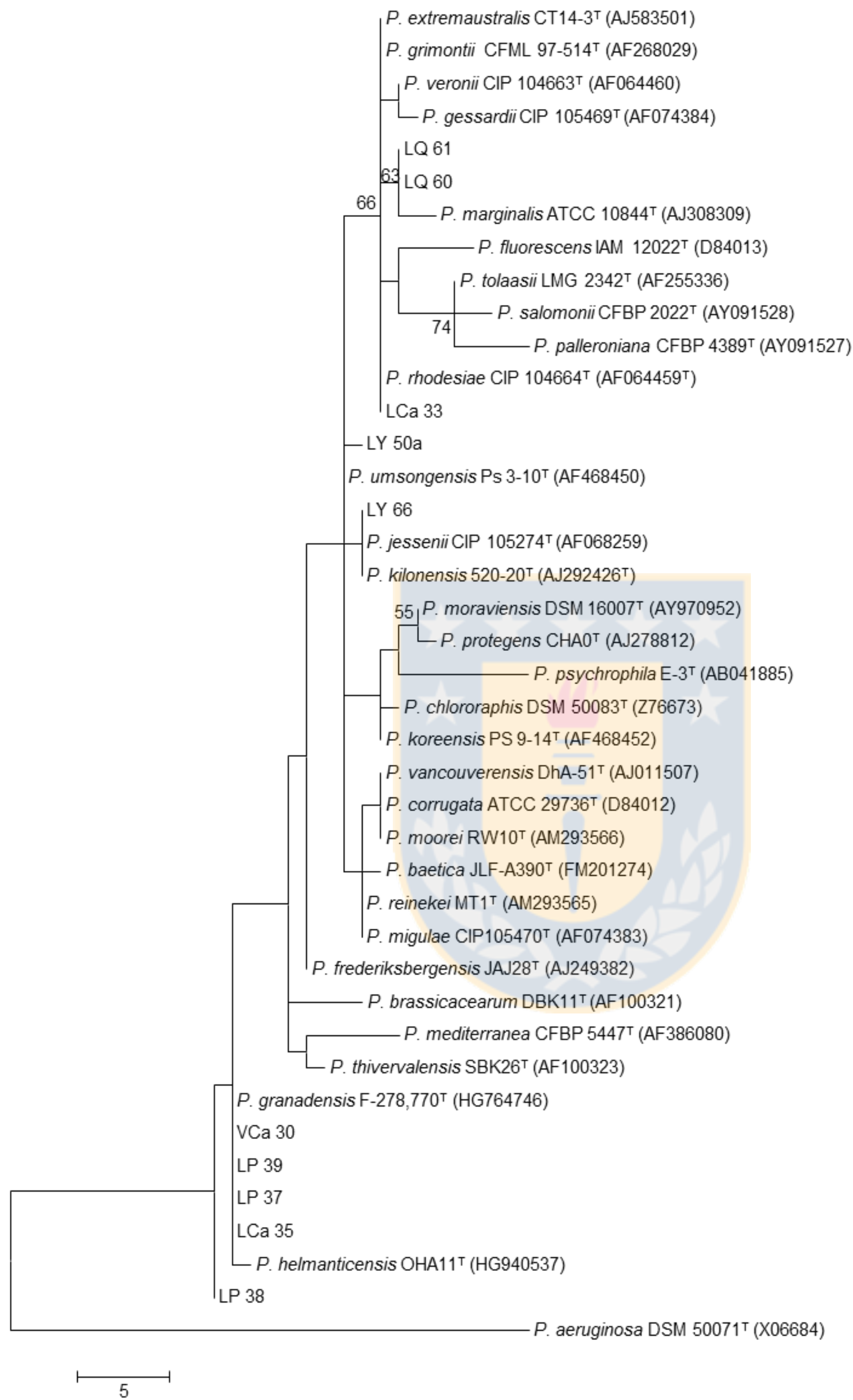

Figure 2: Phylogenetic tree of *Pseudomonas* spp. strains based on 16S rRNA sequences. The tree was constructed using the Maximum Parsimonium method. To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method.

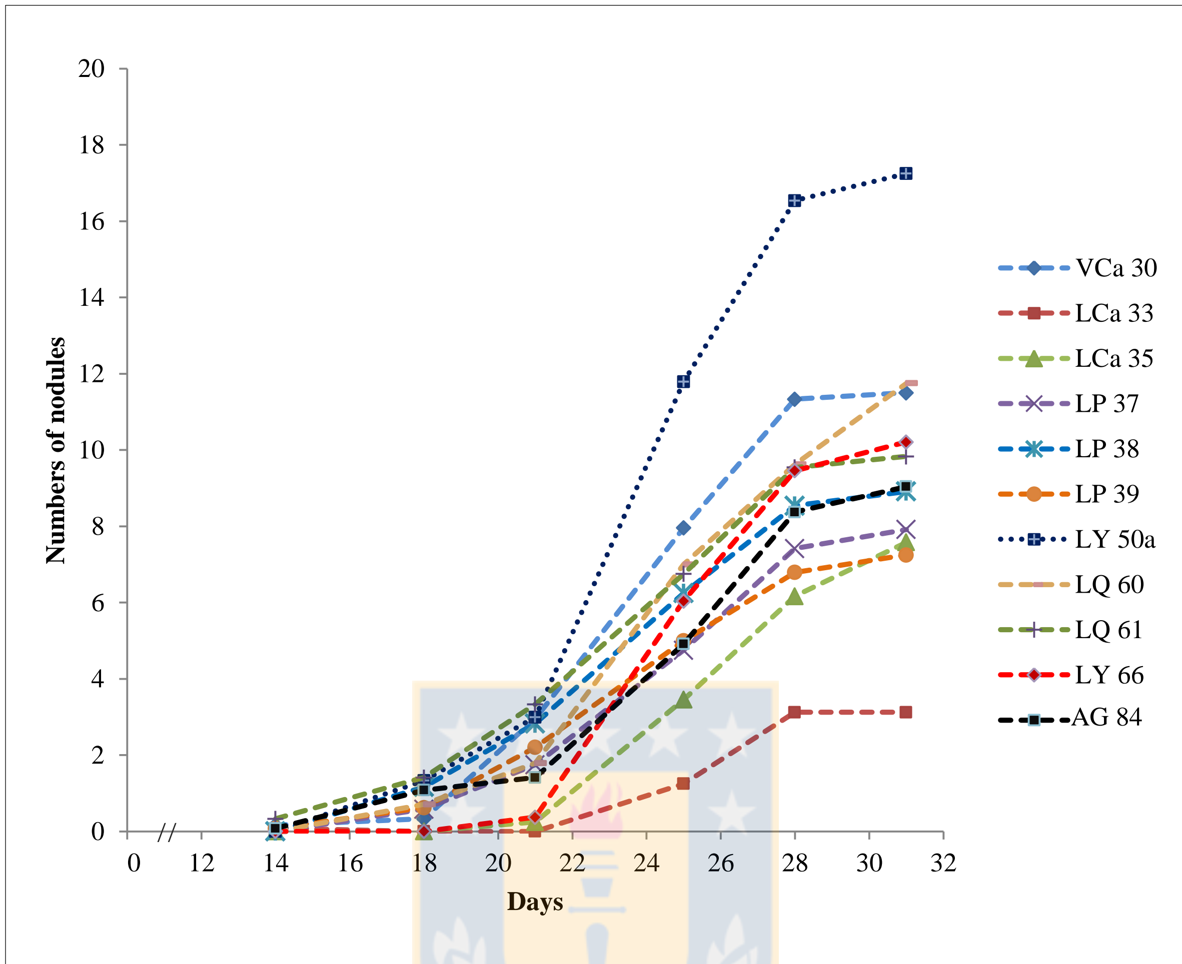

Figure 3: Effect of inoculation of PGPR and rhizobia in the total number of nodules in the time.

Table 1. Selected lentil rhizobacteria strains geographic origin and characteristics of IAA production, ACC deaminase activity and compatibility with rhizobia.

Strain	Geographical origin	Host	IAA Production (µg mL$^{-1)}$ ± EE	ACC deaminase activity	Compatibility
VCa30	El Nogal, Chillán	*V. faba* +	7,77 ± 2,44	+	+
LCa 33	El Nogal, Chillán	*L. culinaris* +	5,62 ± 1,59	-	+
LCa 35	El Nogal, Chillán	*L. culinaris* +	8,85 ± 0,12	-	+
LP 37	Rincomavida, Portezuelo	*L. culinaris* +	16,05 ± 1,54	-	+
LP 38	Rincomavida, Portezuelo	*L. culinaris* +	6,41 ± 0,03	+	+
LP 39	Rincomavida, Portezuelo	*L. culinaris* +	6,91 ± 0,32	-	+
LY 50a	Los Quillayes, Yumbel	*L. culinaris* +	8,73 ± 1,09	+	+
LQ 60	Quitripin, Quirihue	*L. culinaris* *	2,26 ± 1,62	+	+
LQ 61	Quitripin, Quirihue	*L. culinaris* *	3,81 ± 0,65	+	+
LY 66	Los Quillayes, Yumbel	*L. culinaris* *	3,20 ± 0,71	+	+

*Endorhizosphere. + Exorhizosphere. *V.*: *Vicia*. *L.*: *Lens*. Fuente: Datos obtenidos de esta investigación.

Table 2: Effect of inoculation of *Rhizobium leguminosarum* bv. *vicieae* AG-84 and PGPR in the appearance of nodules, number of total nodules and plant dry weight.

Cepa	AUNPC**	Nodule Score	Nodule number	Dry weight (mg)
VCa 30	88,74 ab	38,25 ab	53,25 a	211,0 a
LCa 33	20,74 c	10,50 c	22,75 a	41,3 b
LCa 35	39,81 bc	33,75 ab	56,00 a	217,5 a
LP 37	56,52 bc	18,75 bc	50,75 a	212,3 a
LP 38	75,07 b	29,50 abc	46,50 a	186,5 a
LP 39	60,90 bc	13,00 c	30,75 a	202,5 a
LY 50a	132,01 a	45,75 a	46,00 a	192,8 a
LQ 60	79,12 b	22,00 b	70,50 a	217,0 a
LQ 61	82,86 ab	27,75 abc	51,00 a	212,5 a
LY 66	64,70 bc	30,50 abc	51,25 a	210,5 a
AG 84*	71,61 bc	22,25 bc	43,25 a	217,3 a
cv	50,41	53,88	40,7	15,92

Different letters represent statistically significant differences (P ≤ 0.05) LSD test.
* Control- rhizobia; ** Area under the nodulation progress curve. Fuente: Datos obtenidos de esta investigación.

SUPPLEMENTARY MATERIAL

Strain	Geographical origin	Host	Production IAA ($\mu g\ mL^{-1}$) ± EE	ACC deaminase activity	Compatibility
VCa 1	El Nogal, Chillán	*V. faba* * +	5,28 ± 1,08	+	-
VCa 2	El Nogal, Chillán	*V. faba* * +	4,48 ± 2,89	-	+
VCa 3	El Nogal, Chillán	*V. faba* * +	4,75 ± 1,11	-	-
LCa 4	El Nogal, Chillán	*L. culinaris* * +	4,29 ± 2,50	-	-
LCa 6	El Nogal, Chillán	*L. culinaris* * +	7,45 ± 3,74	-	-
LP 8	Rincomavida, Portezuelo	*L. culinaris* * +	2,07 ± 0,65	-	-
LP 9	Rincomavida, Portezuelo	*L. culinaris* * +	0,40 ± 0,37	-	-
LP 11	Rincomavida, Portezuelo	*L. culinaris* * +	3,25 ± 0,88	-	-
LP 12	Rincomavida, Portezuelo	*L. culinaris* * +	1,85 ±0,91	-	-
LP 13	Rincomavida, Portezuelo	*L. culinaris* * +	2,93 ± 0,84	+	-
LP 14	Rincomavida, Portezuelo	*L. culinaris* * +	1,36 ± 0,65	-	-
LP 15	Rincomavida, Portezuelo	*L. culinaris* * +	6,39 ± 1,74	+	-
LP 16	Rincomavida, Portezuelo	*L. culinaris* * +	2,93 ± 0,80	-	-
LQ 17	Quitripin, Quirihue	*L. culinaris* * +	1,87 ± 0,68	-	-
LQ 18	Quitripin, Quirihue	*L. culinaris* * +	2,77 ± 0,35	-	+
LQ 19	Quitripin, Quirihue	*L. culinaris* * +	4,84 ± 0,99	-	-
LQ 20	Quitripin, Quirihue	*L. culinaris* * +	4,82 ± 0,40	-	-
LQ 21	Quitripin, Quirihue	*L. culinaris* * +	0,67 ± 0,61	-	+
LQ 22	Quitripin, Quirihue	*L. culinaris* * +	2,51 ± 1,35	-	+
LQ 23	Quitripin, Quirihue	*L. culinaris* * +	2,91 ± 0,06	+	-
LY 25	Los Quillayes, Yumbel	*L. culinaris* * +	0,57 ± 0,27	-	-
LY 26	Los Quillayes, Yumbel	*L. culinaris* * +	1,74 ± 1,07	-	-
LY 27	Los Quillayes, Yumbel	*L. culinaris* * +	1,16 ± 0,67	-	-
LY 28	Los Quillayes, Yumbel	*L. culinaris* * +	4,80 ± 2,05	-	-
VCa29	El Nogal, Chillán	*V. faba* +	3,64 ± 0,61	-	+
VCa30	El Nogal, Chillán	*V. faba* +	7,77 ± 2,44	+	+
LCa 31	El Nogal, Chillán	*L. culinaris* +	1,99 ± 0,17	-	+
LCa 32	El Nogal, Chillán	*L. culinaris* +	1,72 ± 0,68	+	+
LCa 33	El Nogal, Chillán	*L. culinaris* +	5,62 ± 1,59	-	+
LCa 35	El Nogal, Chillán	*L. culinaris* +	8,85 ± 0,12	-	+
LP 36	Rincomavida, Portezuelo	*L. culinaris* +	3,12 ± 0,43	-	+
LP 37	Rincomavida, Portezuelo	*L. culinaris* +	16,05 ± 1,54	-	+
LP 38	Rincomavida, Portezuelo	*L. culinaris* +	6,41 ± 0,03	+	+
LP 39	Rincomavida, Portezuelo	*L. culinaris* +	6,91 ± 0,32	-	+

Strain	Geographical origin	Host	Production IAA (μg mL$^{-1)}$ ± EE	ACC deaminase activity	Compatibility
LP 40	Rincomavida, Portezuelo	*L. culinaris* +	3,31 ± 2,81	-	+
LQ 45	Quitripin, Quirihue	*L. culinaris* +	3,20 ± 1,29	-	+
LQ 46	Quitripin, Quirihue	*L. culinaris* +	3,41 ± 0,63	-	+
LY 47	Los Quillayes, Yumbel	*L. culinaris* +	5,00 ± 2,14	-	+
LY 48	Los Quillayes, Yumbel	*L. culinaris* +	6,20 ± 3,49	-	+
LY 49	Los Quillayes, Yumbel	*L. culinaris* +	4,61 ± 1,45	-	+
LY 50a	Los Quillayes, Yumbel	*L. culinaris* +	8,73 ± 1,09	+	+
LY 50b	Los Quillayes, Yumbel	*L. culinaris* +	2,20 ± 0,72	+	+
LY 51	Los Quillayes, Yumbel	*L. culinaris* +	2,26 ± 0,74	-	+
LY 53	Los Quillayes, Yumbel	*L. culinaris* +	4,23 ± 1,09	-	+
LCa 54	El Nogal, Chillán	*L. culinaris* *	4,48 ± 0,99	-	+
LP 55	Rincomavida, Portezuelo	*L. culinaris* *	4,34 ± 0,18	-	+
LP 56	Rincomavida, Portezuelo	*L. culinaris* *	4,36 ± 0,22	-	+
LP 57	Rincomavida, Portezuelo	*L. culinaris* *	1,20 ± 0,45	-	+
LP 58	Rincomavida, Portezuelo	*L. culinaris* *	4,25 ± 0,34	-	+
LP 59	Rincomavida, Portezuelo	*L. culinaris* *	3,71 ± 0,65	-	+
LQ 60	Quitripin, Quirihue	*L. culinaris* *	2,26 ± 1,62	+	+
LQ 61	Quitripin, Quirihue	*L. culinaris* *	3,81 ± 0,65	+	+
LQ 62	Quitripin, Quirihue	*L. culinaris* *	5,17 ± 1,32	-	+
LY 63	Los Quillayes, Yumbel	*L. culinaris*. *	1,66 ± 0,88	-	+
LY 64	Los Quillayes, Yumbel	*L. culinaris* *	4,56 ± 0,99	-	+
LY 65	Los Quillayes, Yumbel	*L. culinaris* *	2,45 ± 0,84	-	+
LY 66	Los Quillayes, Yumbel	*L. culinaris* *	3,20 ± 0,71	+	+
AG 40	Buchupureo	*A. tenella* *	0,26 ± 0,10	+	-
AG 45	Termas de Chillán	*A. emarginata* *	0,84 ± 0,83	+	-
AG 46	Termas de Chillán	*A. emarginata* *	2,22 ± 2,17	+	-
AG 49	Shangrila	*A. emarginata* *	0,11 ± 0,02	+	+
AG 50	Shangrila	*A. emarginata* *	0,70 ± 0,70	+	-

*Endorhizosphere. + Exorhizosphere. *V.*: *Vicia*. *L.*: *Lens*. *A.*: *Adesmia*. Fuente: Datos obtenidos de esta investigación.

CONCLUSIONES GENERALES

Cepas benéficas aisladas desde la endo y exorizosfera de lenteja obtenidas a partir de suelos pobres, con deficiencias nutricionales e hídricas del secano de la región del Biobío, Chile, sintetizan diversos compuestos promotores de crecimiento de las plantas. En esta investigación se obtuvieron diversos aislados con potenciales habilidades promotoras y se seleccionó una cepa, LY50a, para ser utilizada promisoriamente como inoculante para mejorar el crecimiento y la penetración temprana del rizobio en las raíces, a través de la síntesis de AIA de las PGPR y de la actividad de la enzima ACC deaminasa. La coinoculación permitirá mejorar y anticipar la nodulación, aprovechando los beneficios de la fijación biológica de nitrógeno y promoviendo la tolerancia a estrés en el cultivo de la lenteja, resultando en una infección más eficiente y competitiva.