RESEARCH ARTICLE

Serratia strains isolated from the rhizosphere of raulí (*Nothofagus alpina*) in volcanic soils harbour PGPR mechanisms and promote raulí plantlet growth

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Abstract

Raulí is one of the most emblematic tree species of the Chilean temperate forests. Due to the high quality wood, this tree has been used for furniture and handicrafts manufacturing, which has positioned raulí as one of the most important commercial timber species in Chile. Currently, the international market demands sustainable production system for forest production, more specifically in plantlets production. In this regard, plant growth-promoting rhizobacteria (PGPR) inoculants may enhance the growth and survival of plantlets in nurseries, which means an increase in the effectiveness of replanting operations. Therefore, the aim of the present study was to isolate, characterize and screen rhizosphere-associated bacteria with PGPR potential, isolated from raulí that growth in volcanic soils in southern Chile. A total of 1,261 bacterial strains were isolated from different volcanic soils. Out of 1,261 isolates, 100 were selected based on their high levels of indole acetic acid (IAA) production. These isolates were then subjected to screening for 1-aminocyclopropane-1-carboxylic acid deaminase activity, and their ability to fix nitrogen was determined. From the 100 selected isolates, 7 were chosen for producing the highest amount of IAA to continue with genetic characterization based on their 16S rRNA gene sequences. These 7 isolates were characterized as members of the *Serratia* genus and were used to develop multi-strain inoculant mixtures. Later, a nursery study followed to determine the effect of inoculation with the *Serratia* strains on the growth of RA88 raulí clone plantlets. The nursery experiment demonstrated that *Serratia* strains have the potential to increase the root collar diameter, height, relative chlorophyll content, biomass and nitrogen content of raulí plantlets. The study concluded, that *Serratia* strains have the potential to be used as biofertilizers to increase plant growth in nursery conditions.

Keywords: Rhizobacteria, volcanic soil, plant nursery, indole acetic acid, bio-inoculant, forestry, root development.

1. Introduction

Nothofagus alpina Poepp. et Endl. Oerst. (= N. nervosa) commonly known as raulí, is an endemic tree species of subantarctic deciduous-temperate forests (Donoso *et al.*, 2006; Beltran *et al.*, 2017). Raulí is a species that can reach up to 3 m in diameter and 35-40 m in height. In the Andes Mountains the natural distribution of raulí is between 35° 13'S and 37° S. Raulí forest ecosystems are naturally located on soils formed from the deposition of volcanic ash on volcanic rock substratum (Donoso *et al.*, 2006). In southern Chile, volcanic soils (andisols) are the predominant soils that support the bulk of agriculture and forestry production. Andisols are characterized by low pH (4.8–5.8) and a high content of organic matter (Valle and Carrasco, 2018). Among native species, raulí appears to have the greatest potential for commercial use. Due to its rapid growth and wood properties, there is growing demand for this species for use in afforestation, reforestation and enrichment. Furthermore, the high quality of raulí wood is ideal for building and making furniture and handicrafts. Overall, raulí has the potential to become one of the most important commercial forestry species in Chile (Beltran *et al.*, 2017).

Plantations of new raulí forests are becoming more prevalent. Specifically, raulí plantlets are grown in plant nurseries until they reach an appropriated size and root development to be successfully transplanted to their final location in andisols. The productivity of plant nurseries depends on the time that the seedlings require to be ready to be transplanted, and therefore the use of chemical products to promote the first tree development stages is quite common. Currently, there is a need to develop nursery production systems that are friendly to the environment (cleaner technologies). One alternative to the application of chemical fertilizers and pesticides includes the use of plant growth-promoting rhizobacteria (PGPR). Inoculation of agricultural crops with PGPR has been done for many years with encouraging results. Despite of this, research on the application of PGPR to tree species is very limited except for possible use in diseases control in forest nurseries (Chanway, 1997; Lucy *et al.*, 2004; Karličić *et al.*, 2016). Moreover, most of the studies on tree growth promotion focus on gymnosperm species (Lucy *et al.*, 2004).

Currently, there is an increasing interest in the use of beneficial microorganisms as inoculants for nursery tree seedlings or plantlets (Chanway, 1997; Karličić *et al.*, 2016) due to the positive results obtained in agriculture and horticulture, and due to the low cost, environmental friendliness, and easy application of microbial inoculations (Chanway, 1997; Kavino *et al.*, 2010). Recent studies have shown that PGPR inoculants can exert beneficial effects on tree plantlets or seedlings increasing their survival in nurseries, increasing their stress tolerance, and improving the effectiveness of forest replanting operations (Kavino *et al.*, 2010; Karličić *et al.*, 2016). However, there is little information on the effect of PGPR inoculation on the growth and nutrient accumulation of native forest plantlets. There are several possible mechanisms through which PGPR can affect forest plant growth and development (Lucy *et al.*, 2004; Karličić *et al.*, 2016; Zavattieri *et al.*, 2016), for example, production of indole acetic acid and atmospheric nitrogen fixation have stimulated the growth of *Ilex paraguariensis* seedlings (Bergottini *et al.*, 2015), *Cocos nucifera* seedlings (George *et al.*, 2013) and *Eucalyptus nitens* seedlings (Angulo *et al.*, 2014). These studies clearly suggested the potential of PGPR as a pre-plantation nursery treatment.

PGPR strains are broadly distributed among many genera including *Azoarcus*, *Azospirillum*, *Bacillus*,

Burkholderia, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas* and *Serratia* (Martinez-Viveros *et al.*, 2010). Members of the genus *Serratia* exist in a wide range of habitats and have diverse ecological functions such as pathogenic, mutualistic, and antagonistic associations (Grimont and Grimont, 2006; Petersen and Tisa, 2013). The ubiquity of *Serratia* is largely attributed to the variety of compounds that they release into the environment (Petersen and Tisa, 2013). *Serratia* are Gram-negative, facultative anaerobic, rod-shaped bacteria of the Enterobacteriaceae family (Petersen and Tisa, 2013). Different species belonging to the *Serratia* genus are well-known PGPR, and strains have been isolated from the rhizosphere of crops. Plant-associated *Serratia* improve the health and development of their host plant by several mechanisms such as by producing indole acetic acid (Koo and Cho, 2009; Dastager *et al.*, 2011; George *et al.*, 2013), by fixing nitrogen (George *et al.*, 2013), by 1-aminocyclopropane-1-carboxilate deaminase activity (George *et al.*, 2013), by solubilizing phosphate (Dastager *et al.*, 2011; George *et al.*, 2013), by producing siderophores (Koo and Cho, 2009; Dastager *et al.*, 2011) and by inducing systemic resistance of the host plant (Singh and Jha, 2016). A positive role for *Serratia* species in growth promotion have been demonstrated on several plant species, including black pepper (Dastager *et al.*, 2011), corn (Koo and Cho, 2009), wheat (Singh and Jha, 2016) and coconut palms (George *et al.*, 2013).

In view of the above considerations, *Serratia* species have great potential as plant inoculants. However, to the best of our knowledge, there are no studies to date that focus on indigenous *Serratia* strains as inoculants for improving growth of native tree species with forestry importance in volcanic soils. In this context, the objective of the present study was the isolation, characterization, and selection of bacteria from raulí rhizosphere for their biotechnological potential as

PGPR. Subsequently, we selected the *Serratia* strains with the best plant growth promoting (PGP) potential to evaluate the effects of direct inoculation on the growth of raulí plantlets in nursery conditions.

2. Materials and Methods

2.1. Soil sampling and analysis of chemical properties of the rhizosphere of volcanic soils

Rhizospheric soil samples were collected from raulí grown in different volcanic soils from Los Ríos Region (Table 1). Three independent samples were collected from each soil series. The chemical properties of the rhizospheric soil samples, such as pH, total nitrogen (Nt), phosphorus availability (Olsen P), soil organic matter (SOM) and aluminium (Al) saturation, were analysed using the methods revised by Sadzawka *et al.* (2006) and officially recommended for Chilean soils. Soil pH was measured in 1:2.5 soil/deionized water. Ni was extracted with 2M KCl and NO_3^--N was determined with Devarda alloy distillation methods. P was extracted by the Olsen bicarbonate method and analysed by the Murphy and Riley method. SOM was estimated by wet digestion with a modified Walkley-Black procedure. Al saturation was calculated as a percentage of the cation exchange capacity.

2.2. Isolation, screening of plant growth promoting traits, and selection of indole acetic acid producing rhizobacteria

2.2.1. Isolation of rhizobacteria

The rhizobacterial strains were isolated via serial dilution in three common types of agar media: nutritive agar (NA), Jensen agar (JA) and soil extract agar (SEA). Petri plates were incubated 4–7 days at 22 ± 2 °C in the dark. After the incubation period, bacterial

colonies developed were chosen based on their colony morphology and pigmentation; approximately 50 colonies were isolated from each type of agar media for each of the three samples taken from each soil series. Bacterial cultures were stored in nutritive broth with 30% glycerol at -80 °C until further use. Subsequently,

in vitro screening for plant growth-promoting traits such as the production of indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid deaminase (n) activity, and biological nitrogen fixation (BNF) were carried out (George *et al.*, 2013; Etesami *et al.*, 2015).

Table 1. Sample site characteristics

Volcanic soil series	Order	*N. alpina* management	Coordinates	m.a.s.l.	Soil age[a]
Pelchuquín	Andisol	Plantation	Lat: -39.609717° S Long: -73.073683°	27	+++
San José	Andisol	Plantation	Lat: -39.549141° S Long: -73.071677°	23	+++
Malihue	Andisol	Plantation	Lat: -39.544206° S Long: -72.324136°	287	++
Liquiñe	Andisol	Native forest	Lat: -39.837415° S Long: -71.961761°	455	+
Los Ulmos	Ultisol	Plantation	Lat: -39.7510° S Long: -73.1524°	118	+++

a + young, ++ intermediate and +++ old. M. a. s. l.: Meters above sea level.

2.2.2. Determination of indole acetic acid production

The first method used to screen the isolated rhizobacterial production of indole acetic acid was colorimetric quantification of indolic compounds. The ability of the isolates to produce indole compounds was detected in nutritive broth supplemented with L-tryptophan (5 mM) (Martínez *et al.*, 2011). Qualitative detection and quantitative estimation of indole compounds were accomplished using the standard methods described by Patten and Glick (2002). Quantitative estimation was carried out in 15 mL tubes. After incubation (48 h at 30 °C with shaking), bacterial cells were removed from the culture medium by centrifugation at 10,000 × g for 10 min. 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (150 mL of concentrated

H_2SO_4, 250 mL of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) and incubated at room temperature for 30 min (Patten and Glick, 2002). Appearance of a red colour indicated indole compound production. The colour intensity was determined by measuring the optical density at 530 nm using a spectrophotometer (Metertek SP-830). The concentration of indole compounds was determined by comparison with a standard curve prepared with known concentrations of IAA (Sigma-Aldrich). The amount of indole compounds produced was expressed as $\mu g \ mL^{-1}$. Three independent replicates of each isolate were analysed. All isolates were ranked based on their *in vitro* indole compound production.

The second method used to confirm and quantify IAA production was high performance liquid chromatography (HPLC) with fluorescence detection

(Dastager *et al.*, 2011; Angulo *et al.*, 2014). In order to evaluate specific IAA production, this methodology was applied to the 100 highest indole compound producing strains. A Shimadzu (Japan) HPLC instrument equipped with a variable-wavelength UV spectrometer was used. After cultivating the strains, the supernatant was filtered through 0.22 micron filters. Chromatographic separation was then performed by injecting a 20 μL aliquot into a C18 reverse phase column (Supelco, Bellefonte, PA, USA). The fluorescence detector was set with an excitation wavelength (Ex) of 280 nm and an emission wavelength (Em) of 360 nm. A 60:40 mixture of methanol and acetic acid was used as the mobile phase. This was supplied at a flow rate of 1 mL min^{-1} and at a temperature of 23 ºC. Finally, the concentration of IAA at each sample was quantified by obtaining the area of the peaks and by interpolation with an IAA calibration curve.

2.2.3. Determination of ACC deaminase activity

The ability of the strains to hydrolyse 1-aminocyclopropane-1-carboxylic acid (ACC) was assayed according to Penrose and Glick (2003) and Viscardi *et al.* (2016). Two methodologies were applied. First, bacterial isolates were screened for ACC deaminase activity by plating on M9 minimal medium containing ACC as the sole nitrogen source. Second, ACC deaminase activity was determined by measuring the concentration of α-ketobutyrate. Briefly, bacterial cells were grown in M9 minimal medium broth containing ACC as the sole nitrogen source. The amount of α-ketobutyrate in the supernatant was determined by HPLC–PAD (Waters Alliance 2690 HPLC system, Waters 2996 PDA) after centrifugation at 3000 rpm for 10 min. The analyses were carried out using a C8 column (Phenomenex, Luna C8, 250 × 4.6 mm, 5 μm, 100A)

at 30 °C, using a phosphate buffer mobile phase (50 mM, pH 3.0). The flow rate was 1.0 mL min^{-1}. Quantification was carried out at 220 nm by external calibration using pure α-ketobutyrate (Sigma).

2.2.4. Potential capacity to fix biological nitrogen

To evaluate the potential capacity to fix nitrogen, two indirect methods were utilized. Biological nitrogen fixation (BNF) was tested on nitrogen-free medium (George *et al.*, 2013). Bacterial isolates were further screened by plating on Jensen medium without a nitrogen source and incubating at 28 ± 2 °C for 48 h. Growth on the medium reflected the potential ability of bacteria to fix atmospheric nitrogen. Furthermore, the BNF ability of the isolates was assessed by *nif*H molecular detection using polymerase chain reaction (PCR) according to Wartiainen *et al.* (2008). Briefly, PolF/PolR and PolFI/AQER primers designed for soil diazotrophs were used in PCR to molecularly detect the *nif*H gen. In the first PCR reaction, a 370 base pair fragment of the *nif*H gene was amplified using the PCR primers PolF and PolR. In the second PCR, the PCR primers PolFI/AQER were used. The PCR amplifications were carried out with reagents supplied with GoTaq® Flexi DNA Polymerase (Promega Co.) as follows: a hot-start was performed at 95 °C for 10 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C followed by a final extension of 10 min at 72 °C. PCR products were analysed on a 1% agarose gel (LE Agarose, Cleaver Scientific Ltd., USA) stained with GelRed™ (Biotium Inc., USA) and visualized under LED light.

2.3.   Genetic   identification   and   phenotypic characterization of selected IAA-producing rhizobacteria

Isolates were ranked based on *in vitro* IAA production, and 7 superior rank isolates were selected and

characterized using common molecular biology methods. To study the molecular diversity of the IAA-producing rhizobacteria, all selected isolates were subjected to genotyping, identification, and phenotypic characterization.

2.3.1. Genotyping by ERIC-PCR analysis

In order to avoid further analysis of strains with the same genotype, the isolates were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) technique following the Houf *et al.* (2002) protocol. Patterns with at least one different band were considered as different genotypes. The cluster analysis of band patterns was carried out with the Phoretix 1D analysis software (TotalLab Ltd.).

2.3.2. Identification by 16S rRNA gene amplification and sequencing

Selected IAA-producing strains were genetically identified according to 16S rRNA gene sequencing (Coenye *et al.*, 1999). Briefly, genomic DNA was extracted from bacterial cultures grown overnight at 30 °C in nutritive broth. Amplification of the 16S rDNA gene was performed by PCR using the internal primers described by Coenye *et al.* (1999). The 16S rDNA fragments were sequenced by Macrogen (Seoul, Korea). Isolates were identified using 16S rRNA sequence data from the EzTaxon server (http://www.ezbiocloud.net/eztaxon). Nucleotide sequences obtained in this study have been submitted to GenBank under the following accession numbers (MF716551 to MF716557). Phylogenetic analyses with the 16S rDNA sequences of the seven bacterial isolates were conducted using the software MEGA 6 (http://www.megasoftware.net/).

2.3.3. Phenotypic characterization of selected IAA-producing rhizobacteria

Morphophysiological and biochemical characteristics of selected IAA-producing rhizobacteria were examined according to MacFaddin (2000). Specifically, the following traits were determined: Gram staining, development in Salmonella-Shigella culture media (Merck, Darmstadt, Germany), development in MacConkey media (Becton, Dickinson and Company, Le Pont de Claix, France), development at different temperatures 22 °C, 37 °C and 42 °C, haemolysis on sheep blood agar plates, and fermentation of lactose, glucose, arabinose and sorbitol (1% each). Catalase activity was evaluated by adding a 3% H_2O_2 solution and observing the reaction within 5 s. Oxidase activity was determined with Bactident Oxidase strips (Merck).

2.4. Nursery experiment with IAA-producing bacteria

This experiment was carried out in the shadehouse of the nursery at Centro Experimental Forestal (CEFOR), Universidad Austral of Chile, between autumn and spring 2015. A nursery assay was conducted to determine the effect of mixtures of potential PGPR on the growth of raulí plantlets (RA88 clone). Plantlets were two months old and obtained through vegetative propagation. Raulí plantlets were grown in nursery substrate: 50% peat, 50% perlite, 3 g L^{-1} Basacote® Plus (COMPO, Germany) and 160 gm^{-3} Pomarsol® Forte 80% WG (Bayer CropScience).

Selected rhizobacterial strains were -mixtures with different combinations of plant growth promoting traits. Four different treatments were used: control non-inoculated, mix 1, mix 2 and mix 3. Each bacterial mixture contained a different number of strains harbouring different traits related to the promotion of plant growth.

Plantlets were inoculated with a bacterial suspension (10^8 CFU mL^{-1}, 50 mL plant^{-1}) 1, 3, 5 and 7 months after the trial started. Briefly, each strain was grown separately in nutrient broth (Merck) at 23 ± 2 °C in an orbital shaker (150 rev min^{-1}) for 48 h. The cultures were centrifuged in 50 ml sterile plastic tubes at $6000 \times g$ for 15 min. The bacterial cells pellets were re-suspended in saline solution (0.85%) and adjusted to have a final concentration of 10^8 CFU mL^{-1}. For making the bacterial mixtures, equal cell densities (10^8 CFU mL^{-1}) of all individual cultures were mixed together in a 250 mL sterilized flask (Karličić *et al.*, 2017). The control was inoculated with saline solution (0.85%) without bacteria.

Plantlets were subjected to growth evaluation 8 months after the trial started. Plant parameters evaluated were: Biomass (total dry matter, root and shoot dry matter), plant height, root collar diameter (RCD), relative chlorophyll content (RCC), and nitrogen content (Karličić *et al.*, 2017).

2.5. Data analysis

For the plantlet growth experiment treatments were arranged using a completely randomized design. Data were analysed using one-way ANOVA, and a Fisher LSD test was performed in the statistical software Statistica, version 5.0 (SAS Institute, Inc.) to compare means of single variables. Differences were considered significant at $P \leq 0.05$. All the experiments were carried out in triplicate and the values shown are mean ± standard error.

3. Results

3.1. Analysis of the chemical properties of the rhizosphere of volcanic soils

The volcanic ash-derived soils in the sampled raulí plots had different chemical characteristics; the most striking differences included the soil organic matter (13.4-27%) content and aluminum saturation (4.8-37.3%) (Table 2). All soil series had moderately acidic to acid pH values and a low level of available P. The Los Ulmos series had the lowest values of pH, total N and Olsen P.

3.2. Isolation, screening of plant growth promoting traits, and selection of indole acetic acid producing rhizobacteria

Bacteria were isolated from the rhizosphere of raulí from plantations and native forest; culture-dependent standard plate methods were used. Based on differential colony morphologies and pigmentation, a total of 1,261 bacterial strains were isolated with different culture media. The highest number of isolates was obtained with soil extract agar (515 isolates) and nutrient agar (456 isolates). Growth on Jensen medium produced the lowest amount of isolates (290 isolates).

IAA is a plant phytohormone that stimulates the overproduction of root hairs and lateral roots in plants. A well-developed root system is essential for the successful transplant of tree plantlets. Therefore, the production of IAA was the first criterion for the selection of potential PGPR bacteria (Zavattieri *et al.*, 2016). The production of indole compounds was analyzed for the 1,261 isolated strains and the 100 highest indole compound-producing strains. In the 100 selected strains, IAA production ranged from 10.7 to 81.3 μg IAA mL^{-1}. Of these 100 IAA-producing selected strains, 30 strains showed ACCD activity and 36 seemed to have the potential to fix nitrogen (Table 3). In order to further characterize the strains, the 7 isolates that produced the highest amounts of IAA (39 - 49 μg IAA mL^{-1}) were selected for taxonomic identification.

Table 2. Chemical soil characteristics

Variable	unit	Pelchuquin	San José	Malihue	Liquiñe	Los Ulmos
pH (H$_2$O)		5.3 ±0.1 c	5.7 ±0.16 ab	5.5 ±0.3 bc	5.9 ±0.1 a	5.3 ±0.1 c
Nt	(%)	0.72 ±0.02 a	0.62 ±0.04 b	0.77 ±0.07 a	0.49 ±0.03 b	0.39 c
SOM	(%)	24.4 ±1 b	27 ±1.6 a	21 ±2.4 c	13.4 ±1 e	17.4 ±0.8 d
P (Olsen)	(mg kg^{-1})	9 ±0.35 a	5.3 ±1.5 ab	9.7 ±8,3 ab	8.1 ±1.1a	3.9 ±0.42 b
Al sat	(%)	37.3 ±17.1 a	6 ±2.1 b	4.8 ±4.7 b	6.9 ±2 b	31 ±9.4 a

Nt: total nitrogen; SOM: soil organic matter; P(Olsen): Olsen extracted Phosphorus; Al sat: Aluminum saturation. (n=3; ± standard error).

Table 3. Mechanisms related with plant growth promotion in the 100 highest IAA-producing rhizobacteria strains selected

Mechanisms	Number of isolates	Observation
Auxin production	100	10.7 – 81.3 µg mL^{-1}, detection by HPLC
ACC deaminase (qualitative)	76	Bacteria grown in M9 minimal medium agar using ACC as the sole source of nitrogen
ACC deaminase (quantitave)	30	1.94 – 5.74 α-Ketobutyrate µg mL^{-1}, detection by HPLC
Biological nitrogen fixation	36	Detection by PCR using primers targeting the *nif*H gene

3.3. Genetic identification and phenotypic characterization of selected IAA-producing rhizobacteria

In order to understand the molecular diversity of the selected IAA-producing rhizobacteria associated with raulí, DNA fingerprinting employing the ERIC-PCR technique was performed. Differences among selected strains were found in the electrophoretic banding patterns. Specifically, strains were divided into four DNA banding patterns: 1 (Noth 6 and Noth 9), 2 (Noth 4, Noth 5, and Noth 10), 3 (Noth 7) and 4 (Noth 8) (Figure 1).

The 7 strains with the greatest ability to produce IAA were identified using 16S rRNA gene sequence analysis. Results showed that all the IAA-producing rhizobacteria strains belonged to the *Serratia* genus (Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae) (Table 4). Upon analyzing the relationship between the experimental *Serratia* strains and others characterized as belonging to the genus, it was found that two isolates (Noth 6 and Noth 9) are related to *S. myotis* and five (Noth 4, Noth 5, Noth 7, Noth 8 and Noth 10) are related to *S. liquefaciens* group (Figure 2, Table 4). Phenotypic characterization of the 7 selected strains is shown in Table 5. Similarly to the results obtained from the ERIC-PCR analysis (Figure 1), the Noth 4, Noth 5, Noth 7, and Noth 10 strains did not differ phenotypically. In addition, the strains Noth 6 and Noth 9 were phenotypically similar. Conversely, the strain Noth 8 differed from Noth 4, Noth 5, Noth 7 and Noth 10 in its ability to produce the enzyme oxidase and in the ability to produce acid from sorbitol. Noth 8 differed from Noth 6, Noth 7, and Noth 9 due to its utilization of citrate as a sole carbon source and due to the production of the enzyme ACC deaminase (Table 5).
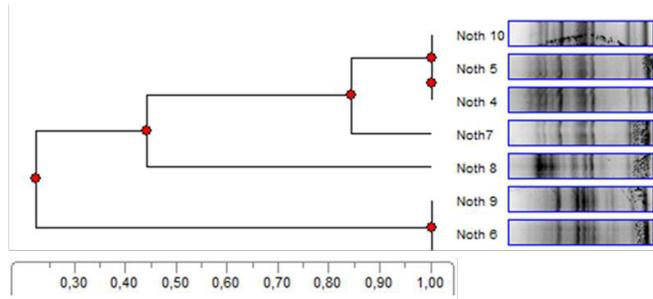
Figure 1. Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR fingerprinting of selected rhizobacteria isolates

Table 4. Identification of selected IAA-producing rhizobacteria strains

Strain	Soil series and medium isolation[a]	Closest relative sequences (accession no.)[b]	Similarity (%)[b]	Accession no.[c]
Noth 4	Los Ulmos SEA	*Serratia liquefaciens* (CP006252)	99.25	MF716551
Noth 5	Malihue NA	*Serratia liquefaciens* (CP006252)	99.25	MF716552
Noth 6	Malihue NA	*Serratia myotis* (KJ739884)	99.86	MF716553
Noth 7	Malihue NA	*Serratia liquefaciens* (CP006252)	99.23	MF716554
Noth 8	Malihue SEA	*Serratia liquefaciens* (CP006252)	99.26	MF716555
Noth 9	San José SEA	*Serratia myotis* (KJ739884)	99.79	MF716556
Noth 10	San José SEA	*Serratia liquefaciens* (CP006252)	99.24	MF716557

[a]JMA: Jensen's Medium Agar, SEA: Soil Extract Agar, NA: Nutritive Agar

[b]The phylogenetic assignment is based on sequence blast against the GenBank database from NCBI (http:// http://www.ezbio-cloud.net/eztaxon/identify).
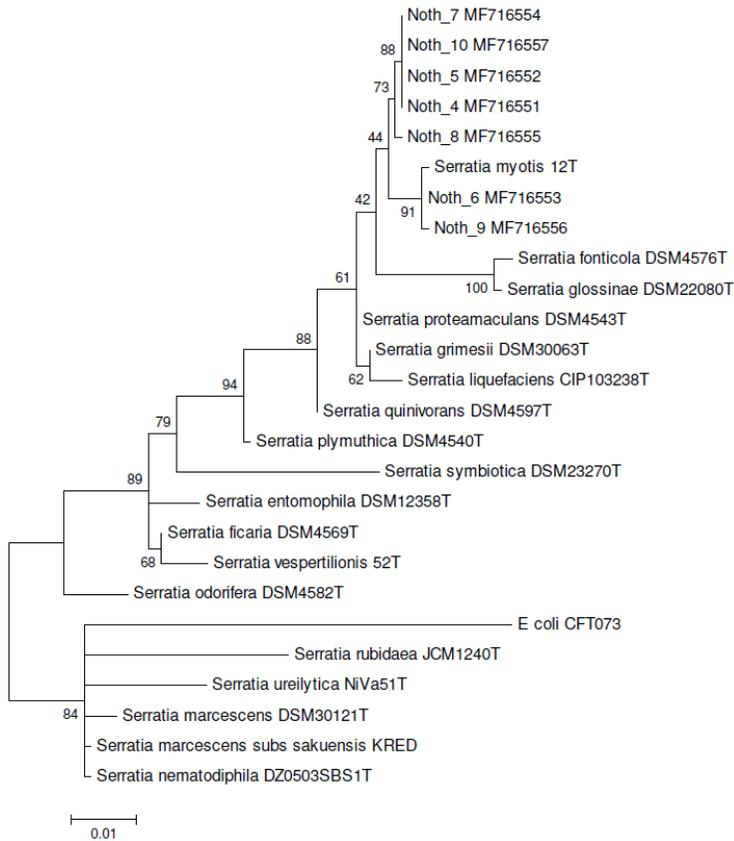
[c]GenBank accession numbers.

Figure 2. Phylogenetic relationship of bacterial strains among *Serratia* species based on nearly full-length 16S rRNA gene sequences. Numbers above branches indicate pseudoreplicate bootstrap values obtained by Maximum Likelihood-Kimura 2 analysis and 1,000 bootstraps

Table 5. Phenotypic characterization of seven selected IAA-producing rhizobacteria isolates from *N. alpina* rhizosphere. + Positive, − negative test

Feature	Noth 4	Noth 5	Noth 6	Noth 7	Noth 8	Noth 9	Noth 10
Morphology in staining of Gram[a]	GNR	GNR	GNR	GNR	GNR	GNR	GNR
Growth 22ºC	+	+	+	+	+	+	+
Growth 37ºC	+	+	+	+	+	+	+
Growth 42ºC	+	+	+	+	+	+	+
Growth in SS agar	+	+	+	+	+	+	+
Growth in MacConkey agar	+	+	+	+	+	+	+
Motility	−	−	−	−	−	−	−
Acid from lactose	−	−	−	−	−	−	−
Acid from glucose	+	+	+	+	+	+	+
Acid from sorbitol	+	+	−	+	−	−	+
Acid from arabinose	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	−	−	+	−	+	+	−
Utilization of Citrate	+	+	−	+	+	−	+
Urease	−	−	−	−	−	−	−
Voges Proskauer	+	+	+	+	+	+	+
Methyl Red	−	−	−	−	−	−	−
DNAsa	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+
Ornithine	+	+	+	+	+	+	+
Hemolysis	β	β	β[b]	β	β	β[b]	β[b]
Plant growth promoting traits							
Indol acetic acid [c]	+	+	+	+	+	+	+
Growth in N-free medium	+	+	+	+	+	+	+
ACC deaminase activity	−	−	+	−	−	+	−

[a]GNR: Gram-negative rod.

[b]Developement of β hemolysis after 48 h.

[c]Test for Indol acetic acid production in peptone broth with 1% tryptophan via Salkowski's method.

3.4. Nursery inoculation experiment with IAA-producing rhizobacteria

In order to study the effects of the *Serratia* strains isolates on plant growth, a nursery experiment was performed. The raulí plantlets were inoculated with 3 bacterial strain mixtures; thus, mix 1 (Noth 4, Noth 5 and Noth 6) contained strains with IAA, ACCD and BNF traits; mix 2 (Noth 7 and Noth 8) contained strains with IAA and BNF traits; and mix 3 (Noth 9 and Noth 10) contained strains with IAA, ACCD and BNF traits. Compared to the control that produced no effect on growth, all of the mixtures significantly stimulated at least some of the plant growth parameters. The growth parameters that were significantly ($P \leq 0.05$) increased after bacterial inoculation compared to the control were: Root collar diameter (52–60%), height (46–49%), relative chlorophyll content (20–28%) and nitrogen content (51–68%) (Table 6). Out of the 3 mixtures tested, the highest stimulation of plant biomass was observed with mix 1. It should be noted that only mix 1 produced significantly greater ($P \leq 0.05$) plantlet total biomass (206%), root dry matter (231%) and shoot dry biomass (191%), compared to the control (Table 6).

Table 6. Effect of bio-inoculation with native *Serratia* mixtures on Raulí (*Nothofagus alpina*) plantlet parameters grown in a nursery

Plant parameters	Control	Mix 1	Mix 2	Mix 3
RCD (mm)	2.1 ±0.11 b	3.4 ±0.26 a	3.5 ±.34 a	3.2 ±0.05 a
Height (cm)	10 ±1 b	14.9 ±1.1 a	14.6 ±1.1 a	14.9 ±1.2 a
RCC (SPAD)	23.1 ±1b	27.8 ±0.7 a	29.6 ±0.7 a	28.5 ±1.6 a
Root dry matter (g)	0.29 ±0.08 b	0.96 ±0.22 a	0.6 ±0.08 ab	0.68 ±0.13 ab
Shoot dry matter (g)	0.49 ±0.06 b	1.43 ±0.33 a	1.01 ±0.14 ab	1.06 ±0.18 ab
Dry matter (g)	0.78 ±0.14 b	2.39 ±0.55 a	1.61 ±0.22 ab	1.75 ±0.32 ab
N content (%)	0.47 ±0.089 b	0.79 ±0.012 a	0.76 ±0.031 a	0.71 ±0.006 a

RCD: Root collar diameter; RCC: Relative chlorophyll content. (n=3; ±standard error).

4. Discussion

In the present study, native bacteria were isolated from the rhizosphere of raulí growing in different volcanic soils, and these bacteria were screened for the presence of putative plant growth-promoting traits. The isolation and identification of native bacteria is an important first step in identifying candidate bacteria with potential PGPR traits adapted to different soil environments. In southern Chile, raulí grow on ash-derived volcanic soils known as andisols, which are characterized by a high level of metal phytotoxic cations (Al^{+3} and Mn^{+2}) in the soil solution (Valle and Carrasco, 2017). These chemical properties of the rhizosphere soil have a relevant influence in the occurrence and performance of culturable rhizobacteria that contain beneficial traits for plant growth (Martínez *et al.*, 2011).

In this study, 1,261 bacteria were isolated from the rhizosphere of raulí growing in plantations and in one native forest in southern Chile. These isolates were screened for well-known traits commonly attributed to PGPR such as IAA production, ACCD activity and BNF. Currently it is well known that IAA production is widespread among soil bacteria that inhabit the rhizosphere of plants, and IAA production promotes growth and development (Egamberdieva *et al.*, 2017; Viscardi *et al.*, 2016); thus, this trait was

our principal criterion for selection. Others have also noted that IAA production can be used for the selection of effective plant growth promoting rhizobacteria (Etesami *et al.*, 2015; Mestre *et al.*, 2017). The main IAA-producing bacteria belonging to the *Aeromonas*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Rhizobium* and *Serratia* genera have been isolated from different rhizospheric soils (Egamberdieva *et al.*, 2017; Koo and Cho, 2009; Martinez-Viveros *et al.*, 2010). The ability of rhizobacteria to produce phytohormones has long been believed to be a major factor affecting plant root growth; specifically, it is thought that these phytohormones allow the plant to extract nutrients from a greater volume of soil (Egamberdieva *et al.*, 2017). Additionally, other studies have shown that IAA produced by PGPR affects tree root morphology (George *et al.*, 2013) and metabolic processes such as photosynthesis, biosynthesis of various metabolites, and resistance to stressful conditions modulated by auxins (Egamberdieva *et al.*, 2017; Karličić *et al.*, 2017). Accordingly, IAA indirectly increases water and nutrient supplies leading to higher root exudation and biomass production in forest species (Egamberdieva *et al.*, 2017; Karličić *et al.*, 2017). Furthermore, it has been shown that IAA triggers the activation of the central energy metabolism of *Escherichia coli* cells (Bianco *et al.*, 2006b) and is also able to enhance protection against various

abiotic stresses such as UV and low pH (Bianco *et al.*, 2006a). From this, we hypothesize that the high levels of IAA production by the rhizobacteria studied here might be related to the defense against stress caused by the low pH and high aluminum concentration in these soils. Overall, our results are in agreement with previous reports that indicate that the production of IAA is a widely distributed trait in culturable bacteria isolated from plants grown in Chilean andisols (Jorquera *et al.*, 2014; Martínez *et al.*, 2011).

Here we found that several of our isolates had the ability to produce IAA, had ACCD activity, and had the potential to fix nitrogen. It is well known that plant-associated bacteria can have multiple plant growth-promoting traits (Martinez-Viveros *et al.*, 2010). It has been reported by Etesami *et al.* (2015) that bacterial isolates with positive IAA and ACC deaminase-producing traits can increase plant colonization. Bacterial IAA together with ACC deaminase increase root surface area and length and thereby provide the plant with access to soil nutrients. Increased nutrient availability is important during situations of plant stress such as during transplantation. Additionally, BNF is an important plant growth promoting trait, and many non-symbiotic N-fixing rhizospheric bacteria belonging to the genera *Azoarcus*, *Azospirillum*, *Burkholderia*, *Gluconacetobacter*, *Pseudomonas* and *Serratia* have been isolated from different soils (Dastager *et al.*, 2011; Martinez-Viveros *et al.*, 2010). Overall, the PGP traits evaluated here could be targeted to aid the successful transplantation of raulí trees in volcanic soils. Specifically, rhizobacteria with these traits could be used to support the growth and development of trees despite the low pH and the high concentration of metals (Al^{3+} and Mn^{2+}) in the soil solution (Karličić *et al.*, 2016; Kavino *et al.*, 2010).

In the present study, the highest IAA-producing bacteria isolated from the rhizosphere of raulí grown in different volcanic ash-derived soil belonged to the genus *Serratia*. ERIC-PCR fingerprinting analysis showed that the *Serratia* isolates grouped into four genotypes. Analysis of the 16S rRNA gene showed that the isolates Noth 6 and Noth 9 can be identified as S. *myotis* and strains Noth 4, Noth 5, Noth 7, Noth 8 and Noth 10 are related the *S. liquefaciens* group. The *S. liquefaciens* group includes three different species -*S. liquefaciens*, *S. grimesii*, *S. proteamaculans*- that are not distinguishable by 16S rRNA sequencing. Therefore, further analysis would be necessary to identify the strains to the species level. On the other hand, phenotypic characterization indicated that all 7 isolates were able to grow in N–free medium, which shows their potential capacity to fix nitrogen. Additionally, Noth 6 and Noth 9 isolates showed ACCD activity. Several reports have shown that *Serratia* species have PGPR potential (Dastager *et al.*, 2011; George *et al.*, 2013). Furthermore, previous studies have identified PGPR strains from rhizosphere of Chilean volcanic soils as belonging to the *Serratia* genus (Jorquera *et al.*, 2014; Martínez *et al.*, 2011). Thus, this group of bacteria have the potential to enhance plant development in this kind of soil.

The evaluation of the plant growth-promoting effects by the rhizobacteria was carried out in a nursery. For the inoculation assay, we selected seven *Serratia* isolates with PGPR traits favoring IAA production, ACC deaminase activity, and BNF. The nursery experiment results showed that when the *Serratia* strains were added to raulí plantlets an increased RCD (56%), height (47.5%), RCC (24%) and N content (59.5%) was found. On the other hand, only plants with mix 1 added had increased root dry matter (2.3 fold), shoot dry matter (1.9 fold) and total dry matter (2 fold). Aligned with these results, George *et al.* (2013) have shown that inoculation with *S. marcescens* increases biomass (total dry weight) in coconut seedlings (19%), cowpea seedlings (104%) and paddy seedlings (60%) compared to non-inoculated controls.

In addition, George *et al.* (2013) report a significant increase in the collar girth (6.2 %) and N uptake (54 %) of coconut seedlings. Plantlet root collar diameter (RCD) is an indicator of plantlet quality, and larger RCDs are usually correlated with higher survival and growth (South *et al.*, 1985). Furthermore, increased RCDs can be related to increases in root biomass. The present study demonstrates that *Serratia* strain inoculation increases plantlet N-content, and this can be attributed to the N fixation ability of these strains. Regardless, direct transfer of N should be measured to verify these results (Bergottini *et al.*, 2015). To our knowledge, this is the first report where rhizosphere raulí-associated *Serratia* strains have been shown to promote growth of raulí plantlets in nursery conditions. Overall, the potential of these rhizobacteria to be implemented as a biofertilizer in nursery management requires further investigation.

5. Conclusions

The present work reveals that raulí grown in volcanic soils contain rhizobacterial populations that harbour traits that promote plant growth. Specifically, these traits include the production of indole acetic acid, 1-aminocyclopropane-1-carboxilate deaminase activity and biological N fixation. The most efficient culturable indole acetic acid-producing rhizobacteria were selected and identified as S*erratia* species. Moreover, this study shows that indigenous *Serratia* strains can enhance raulí plantlet quality in nursery conditions. Thus, these rhizobacteria are promising inoculants for the development of plantlets to transplant into volcanic soils.

Acknowledgements

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