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Aluminum-tolerant bacteria improve the plant growth and phosphorus content in ryegrass grown in a volcanic soil amended with cattle dung manure

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ABSTRACT

In Chilean volcanic soil, crop production often is limited by a combination of the low available P and high concentration of toxic aluminum (Al). In this study we aimed to isolate Al-tolerant plant-growth-promoting bacteria from the rhizosphere and the endosphere of ryegrass grown in acidic Chilean volcanic soil in order to characterize a bacterial consortium capable of contributing to alleviation of Al^{3+} toxicity and supporting plant growth in Andisol. Five strains, i.e. *Klebsiella* sp. RC3, *Stenotrophomonas* sp. RC5, *Klebsiella* sp. RCJ4, *Serratia* sp. RCJ6 and *Enterobacter* sp. RJAL6, were selected based on their capacity to tolerate high Al concentration (10 mM) and to exhibit multiple plant-growth-promoting traits (P solubilization, indole acetic acid production, 1-aminocyclopropane-1-carboxylate deaminase activity, and exudation of organic acid anions and siderophores). Based on the results, we can suggest that selected bacteria could alleviate Al stress by forming Al^{3+}-siderophore complexes. The plant-growth-promoting potential of the bacterial consortium was confirmed in an assay with ryegrass plants. In the treatment with cattle dung manure, the consortium promoted plant growth and the phosphatase activity in the rhizosphere soil. Increased phosphatase activity coincided with elevated P concentration in shoots. Our results suggest that a combination of native Al-tolerant bacteria and cattle dung manure is effective in decreasing Al toxicity and promoting plant growth in Andisols of southern Chile.

1. Introduction

Acidic volcanic soils (Andisols) represent around 60% of agricultural soils in southern Chile. Pasture production in Andisols supported around 56% of beef and 85% of dairy cattle production in Chile in 2014–2015 (ODEPA, 2015). Chilean Andisols are characterized by low pH and low available P concentrations, yet a high total P content (Mora et al., 2009).

Soil acidification is a natural process that can be accelerated over time under intensive agricultural systems. The acidification is increased due to the traditional urea fertilization, which is often used in agricultural systems due to its low cost and high N content (46%). Soil acidification results in an increase in phytotoxic aluminum (Al^{3+}), as demonstrated by the relationship between Al saturation percentage and pH in Chilean Andisols (Mora et al., 2005).

Increased levels of toxic Al of up to 5000 mg kg^{-1} dry weight in Chilean Andisols threaten large areas of pastures in Southern Chile and result in a loss of yield and quality of forage (Mora et al., 2002, 2006). The primary toxic effect of Al^{3+} is a rapid root growth inhibition that limits water and nutrient uptake by plants (Kochian et al., 2004). Therefore, P deficiency and Al^{3+} toxicity coexist in Chilean Andisols and threaten efficient crop production (Mora et al., 2002, 2007).

Several studies involving Al toxicity in plants have been reported. However, very few investigations have been focused on the effect of Al toxicity on soil microorganisms and their tolerance mechanisms (Guida et al., 1991). Thus, improved mechanistic understanding of plant-microbe interactions could contribute to better crop management strategies in Chilean Andisols. Our research group currently is working in P recycling from cattle dung manure and P fractionation using phosphomonoesterase enzymes in order to improve P plant nutrition (Fuentes

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et al., 2009; Menezes-Blackburn et al., 2014). The results may develop a novel agronomic strategy for improving the organic P mineralization from cattle manure applied to soils.

The rhizosphere and endosphere contain a wide variety of bacterial species that carry out functions essential to nutrition, growth and disease suppression in plants (Nannipieri et al., 2003, 2007; Hawkes et al., 2007; Durán et al., 2014). The beneficial effects of bacteria on plant growth have been attributed to the presence of plant-growth-promoting-bacteria (PGPB). The PGPB can i) produce (or regulate the concentrations of) phytohormones, ii) increase availability of nutrients to plants (e.g., release of P, nitrogen fixation, siderophore exudation), and/or iii) control phytopathogens (production of antibiotics and siderophores) (Martínez et al., 2011).

Siderophore production has been related to chelation of Fe^{3+} in Fe-deficient environments to facilitate Fe uptake into microbial cells (Carrano et al., 1996). Aluminum and Fe have a similar ionic radius (54 and 64 pm, Yokel, 2002). Thus, some siderophores can also bind Al and other metals such as copper, zinc, chromium, lead, manganese, cadmium, vanadium, gallium or indium (Baysse et al., 2000; Cornelis 2008). Therefore, we speculated that siderophore-producing bacteria may also harbor mechanisms for Al detoxification such as the chelation of Al^{3+}. In the present study, we isolated and characterized native Al-tolerant bacteria from the rhizosphere and endosphere of ryegrass grown in an volcanic soil and evaluated their effect on growth of ryegrass in the presence of cattle dung manure.

2. Material and methods

2.1. Enrichment and isolation of native bacteria

Bacteria were isolated from the rhizosphere and tissues (root and shoot) of ryegrass (*Lolium perenne*) grown in Andisol (series Freire) located in Barros Arana, Region of La Araucanía. Plants were collected using a cleaned spade to excavate intact roots from soil to a depth of 0–20 cm and were immediately transported to the laboratory in a cooler at 4 °C.

For the isolation of rhizosphere bacteria, soil aggregates were removed from the roots by shaking for 5 min in 50 mL of sterile saline solution (0.85% w/v NaCl). The occurrence of culturable bacteria was examined by plating serial dilutions of the rhizosphere soil on Luria-Bertani (LB) agar according to Vallini et al. (2005).

For the isolation of the endophytic bacteria, the root surface was sterilized by immersion in 80% v/v ethanol (5 min) followed by 4% v/v NaOCl (20 min), and triple rinsing of the root with sterile distilled water (Shimizu, 2011). Root samples were then macerated and homogenized in 1 mL of sterile saline solution (0.85% w/v NaCl). One hundred microliters of homogenized tissue dilutions were spread onto LB agar plates and incubated at 30 °C for 4 days as described by Durán et al. (2014). The efficacy of tissue surface sterilization was confirmed by spreading the last-run rinsing water onto LB agar plates.

Diverse colonies were selected based on their phenotype (size, edge, color, etc.) and genotype as determined by ERIC-PCR technique using the primers set ERIC-1R/ERIC-2 (5′- ATGTAAGCTCCTGGGGATTCAC −3′ and 5′- AAGTAAGTGACTGGGGTGAGCG −3′). The isolated strains showing different genotype were then screened for their plant growth promoting (PGP) traits and Al tolerance.

2.2. Al tolerance and PGP traits

The minimum inhibitory concentration (MIC) of Al in isolated strains was determined on agar plates containing (g L^{-1}): 10 mM of succinic acid, 1 g L^{-1} NH_4C1, 50 g L^{-1} glycerol, 1 g L^{-1} KCl, 0.01 g L^{-1} $CaC1_2 \times 2H_2O$, and 0.87 g L^{-1} K_2SO_4 (Guida et al., 1991). 1 mL L^{-1} of trace element solutions (Poole and Haddock, 1974), glycerophosphate (16.5% w/v) and 1 mg L^{-1} $MgCl_2 \times 6H_2O$ (20% w/v) were autoclaved separately and added aseptically to the sterile medium (pH 4.8). Aluminum was freshly prepared as solution of $Al(NO_3)_3$ at final concentration of 0, 5, 10, 20 and 40 mM. Appropriated dilutions of isolated strains were spread on agar plates and then incubated at 30 °C for 4 days.

The following PGP traits were determined in each of the isolated strains: the capacity to utilize insoluble organic and inorganic phosphorus (P) and to produce phytohormone indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase (ACC) and siderophores. The capacity to utilize P on agar was examined based on the clear halo formation on National Botanical Research Institute' phosphate (NBRIP) growth medium supplemented with tricalcium phosphate ($Ca_3[PO_4]_2$) (Nautiyal, 1999) and phytase screening medium (PSM) containing sodium phytate ($C_6H_6Na_{12}O_{24}P6 \times H_2O$) as a sole P source (Kerovuo et al., 1998). Halo formation was measured as follows: +++, very high capacity; ++, high capacity; +, normal capacity; and −, no capacity.

The IAA production was determined at 535 nm using Salkowski's reagent as described by Patten and Glick (2002), whereas the ACC deaminase production was determined for cultures grown in Dworkin–Foster (DF) salts minimal medium containing ACC compounds as the sole N source (Glick, 2003). The siderophore production was evaluated on agar plates supplemented with chrome azurol S (CAS) reagent as described by Alexander and Zuberer (1991).

Finally, isolated strains were selected according to their tolerance of elevated Al concentration and their expression of multiple PGP traits.

2.3. Exudation of organic acid anions

Exudation of organic acid anions by selected strains was analysed by high performance liquid chromatography (HPLC). The strains were cultivated in LB broth at 30 °C with shaking. After 24, 72 and 96 h of incubation, a 2-mL aliquot was collected and centrifuged at 15,700 × *g* for 1 min to pellet cells; the supernatant was filtered through a 0.22-μm membrane and stored at −21 °C until analysis. Then, 20 μL of diluted filtrate (1:100) was injected to a HPLC machine (HITACHI model Primaide) equipped with a UV–210 nm detector. The organic acid separation was carried out with a RP-18 150833 column (Merck Art.1.50833.001). The operating conditions consisted of 25 mM H_3PO_4 as mobile phase at a constant (isocratic) flow rate of 0.6 mL min^{-1} at 21 °C. Retention time (RT) of each signal was recorded at a wavelength of 210 nm. The software used for HPLC analyses was Primaide System Manager, and the organic acids were identified by comparing their retention times and the peak areas of chromatograms with those obtained from pure standards. All analyses were performed in triplicate.

For selected bacterial strains the 16S rRNA gene was partially sequenced. The 16S rRNA gene fragments were amplified by PCR with the universal bacterial primer sets 27f (5′-AGA GTT TGATCC TGG CTC AG-3′) and 1492r (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Peace et al., 1994). After hot-start at 94 °C for 5 min, PCR amplification was carried out for 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min. The PCR products were purified and sequenced by Macrogen Inc. (Korea). The sequences were deposited under accession nos. from KU697293 to KU697297 and compared with those in the GenBank database.

2.4. Siderophores and Al complexes

As siderophores may not bind only to Fe^{3+}, siderophores from selected strains were also analysed for their capacity to bind to Al^{3+}