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Original article

Evaluation of rhizobacterial isolates from Argentina, Uruguay and Chile for plant growth-promoting characteristics and antagonistic activity towards *Rhizoctonia* sp. and *Macrophomina* sp. *in vitro*

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ABSTRACT

This study was conducted over 3 years in soils of Uruguay and Chile and two years in soils of Argentina. 686 bacterial isolates were phenotypically characterized by testing in relation to the presence of plant growth promoting properties: phosphate solubilization, production of siderophores, starch hydrolysis, production of exopolysaccharides and biological control of *Macrophomina phaseolina* and *Rhizoctonia* spp. In all samples analyzed, the number of Gram-positive bacteria exceeded that of Gram negative. Ten bacterial isolates were selected for their plant growth promoting properties and API Test and 16S rRNA gene (rDNA). Six of these isolates belong to the genus *Pseudomonas*, three to the genus *Bacillus* and one to *Janibacter*. This is the first report of a strain from the genus *Janibacter* with promising plant growth-promoting attributes. The results obtained allow us to improve the microbial germplasm of plant growth promoting bacteria from soils of Chile, Argentina and Uruguay with a view to their potential use in the formulation of mixed inoculants that promote the growth of alfalfa.

1. Introduction

The rhizosphere, defined as the volume of soil adjacent to and influenced by the plant root [1], is of great importance to plant health and soil fertility [2]. This space is limited and it extends to a few millimeters from the root surface [3]. Root secretions, such as amino acids and carbohydrates, constitute a rich source of energy and nutrients for microorganisms, thus, microbial populations are high in this area.

Plant growth-promoting rhizobacteria (PGPR) [4] are non pathogenic beneficial bacteria that play a fundamental role in plant health and nutrition. They can benefit plant growth through diverse mechanisms such as solubilization of mineral phosphates [5–7] and other nutrients, production of growth-regulating compounds [8,9], and prevention against pathogens attacks [10,11].

Alfalfa (*Medicago sativa* L.) participates in a high percentage of the cultivation area in the agricultural-cattle systems of Chile, Uruguay and Argentina. At the present time, it constitutes one of the most important forage resources due to its enormous adaptation to different climates and soils. In Argentina, the highest cultivated area corresponds to the provinces of Córdoba, Santa Fe, Buenos Aires and La Pampa, where water availability and soils with low pH and low levels of soluble phosphates are the main obstacles for an effective biological nitrogen fixation (BNF) [12]. In Chile, it is successfully cultivated in central and northern-central regions, where there are favorable soils and weathers. However, production of this crop is limited in southern and southern-central areas of the country because of acid soils derived from volcanic ashes (andisols), furthermore, these soils contain high concentrations of Al and Mn and a low concentration of P [13,14]. In Uruguay, alfalfa is cultivated in the core area of intensive animal production, where it is in full expansion due to its persistence.

Plagues and diseases can considerably reduce the quality, persistence and nutritious value of forage [15]. Alfalfa is affected by several diseases that attack leaves, stems, crown and roots. Pathogens that attack roots and crown, such as *Macrophomina* spp. and

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Rhizoctonia spp., directly define the longevity or the productive period of alfalfa.

It is of great interest to develop biotechnological products that combine the positive effects of different species of rhizosphere microorganisms (multiple inoculants) in order to benefit plants with a higher capacity to incorporate water and nutrients thus improving their health and yields.

In this work, we analyzed the potential plant growth-promoting traits and the biocontrol capacity of a representative bacterial isolates sample obtained from six pre and post alfalfa planting soils from Chile (Carillanca and Faja Maisan), Uruguay (Punta Espinillo I and II) and Argentina (Manfredi and Balcarce).

Our objective was to determine the proportion and diversity of plant growth-promoting characteristics and the antagonistic ability against *Rhizoctonia* spp. and *Macrophomina* spp. *in vitro* of bacteria isolated from different South American soils, and if these attributes are related to each other and/or to the isolation site.

2. Materials and methods

2.1. Soil and soil sampling

Soil samples were collected from six sites located in Chile, Uruguay and Argentina. The samples from Chile and Uruguay were conducted for three consecutive years (2004–2005–2006), while trials in Argentina corresponded to the years 2004–2005. This was due to climatic factors that influenced the phenological cycle of the crop. The samples processed following the method described by Frioni [16] included soil (preplant sampling) and rhizosphere soil (sampling post seeding). Rhizosphere soil samples were obtained from fields of alfalfa cultivation inoculated with *Sinorhizobium meliloti* B399 (strain recommended by the Instituto Nacional de Tecnología Agropecuaria – INTA for alfalfa inoculation in Argentina) with a dose of approximately of 10^3–10^4 CFU/g seeds) and uninoculated fields. Identical field assays were designed in all three countries. At least two different agro-ecological regions were selected where, during the past five years, no alfalfa or other *S. meliloti* host plant had been planted. All information on different soils is compiled in Table 1.

2.2. Isolation of bacteria and determination of colony forming units

One gram of each soil was suspended into 9 ml of sterile saline solution (9 g l^{-1} NaCl). Serial 10-fold dilutions were performed. Then, a 0.1 ml aliquot was plated onto 25% Tryptic Soy Agar (TSA; Britania Laboratories), in triplicate. Plates were incubated at 28 °C for 24–48 h. Results were expressed as colony forming units g^{-1} soil (CFU g^{-1} soil).

2.3. Selection of bacterial isolates

The first characterization consisted of direct observation of isolated colonies, taking into account color, shape, elevation, margins, diameter, surface, opacity and texture [17]. Colonies showing visible morphological differences were re-isolated on 25% TSA and yeast extract mannitol agar (YEMA) supplemented with congo red [18] to differentiate the colonies of rhizobia (not selected for further testing). The selected strains were conserved at −80 °C in Tryptic Soy Broth (TSB-Britania®, Argentina), amended with 20% glycerol.

2.4. In vitro tests

Each isolate was first subjected to Gram stain and to the complementary 3% KOH test [19]. Isolates were characterized by means of phosphate solubilization [7], siderophore production [20], starch hydrolysis and exopolysaccharide production [21] assays. Also, assays of antifungal capacity were carried out in 25% TSA medium. To this end, a mycelial disc (9 mm diameter) of an actively growing target fungus was placed on the center of a Petri plate and bacterial isolates were inoculated in the periphery. Plates were incubated for 7 days at 28 °C. Inhibition zones were recorded and compared to a growth control (target fungus alone). The strain *Pseudomonas clororaphis* subsp. *aurantiaca* SR1 [22,23] was used as positive control. The phytopathogenic fungus strains were isolated from infected plant tissue and identified by Laboratorio de Micología, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto [24,25].

2.4.1. Phenotypic characterization

Based on the results of the preliminary characterization described above, ten promising isolates were identified to the species level by the API identification system assisted by analytical profile index (API) Plus computer software (bioMe'rieux_SA, Marcy-l'Etoile, France). Gram positive, endospore forming rods were identified to the species level using API 50 CH test strips. Gram positive, short, nonmotile (verified by the SIM (Hydrogen–Sulfide, Indole, Motility) [26], non-spore-forming, strictly aerobic, catalase positive, and oxidase negative rods were identified by using the API Coryne strip. Gram negative rod isolates with only oxidative reaction in OF basal medium [27] were identified using the API 20 NE test strip. The API strip consists of microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests were inoculated with a bacterial suspension which reconstituted the media. After incubation, the metabolic end products were detected by indicator systems or the addition of reagents. The substrate microtubes contained assimilation tests and were inoculated with a minimal medium. If the isolates were capable of utilizing the corresponding substrate, they grew.

2.5. Genotypic characterization of bacterial isolates

2.5.1. DNA extraction from isolates. Partial nucleotide sequences of the 16S rRNA gene and BOX-PCR genomic fingerprints

Ten isolates showing the greatest P and Fe solubilization halos together with the highest antifungal ability were selected for

Table 1
Description of soils from the six sites.

Country/location	% Organic matter	pH	P (ppm)	K	N	Ca	Mg	Na	Soil type	Cropping history
Uruguay/Punta Espinillo 1 (56° 25′ W, 34° 49′ S)	0.7	5.9	27	0.55[a]	ND	8.4[a]	3.4[a]	0.31[a]	ND	Intensive horticulture
Uruguay/Punta Espinillo 2 (56° 25′ W, 34° 49′ S)	1.8	5.5	31	0.80[a]	ND	4.7[a]	4.7[a]	0.13[a]	ND	Intensive horticulture
Chile/Carillanca (72° 40′ W, 39° 06′ S)	19.0	5.7	23	0.53[b]	25[c]	2.99[b]	0.4[b]	0.1[b]	Typic Hapludand	Mixed prairie
Chile/Faja Maisan (72° 55′ W, 39° 05′ S)	17.0	5.4	14	1.23[b]	37[c]	4.52[b]	1.27[b]	0.13[b]	Typic Hapludand	Mixed prairie
Argentina/INTA Manfredi (63° 44′ W, 31° 50′ S)	1.71	6.2	46	ND	0.105[d]	ND	ND	ND	Haplustol Entic	Gramineous crops
Argentina/INTA Balcarce (58° 15′ W, 37° 50′ S)	4.9	6.1	12.8	2.3[a]	0.352[d]	15.5[a]	2.9[a]	0	Petrocalcic Paleudol	Gramineous crops

ND: not determined.
[a] meq 100 g^{-1}.
[b] cmol kg^{-1}.
[c] Inorganic N (ppm).
[d] Total N (%).