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Original article

Biocontrol bacterial communities associated with diseased peanut (*Arachis hypogaea* L.) plants

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ABSTRACT

In the present study, the isolation and characterization of endophytic and epiphytic biocontrol bacteria from peanut plants affected with white mold and root rot are described. Two hundred and sixty three isolates were selected from diseased plants and biocontrol traits analyzed were antibiosis against *Sclerotinia minor* and *Fusarium solani*, siderophore production and similarities in carbon source utilization between bacteria and fungi. Diversity and sequence analysis of potential biocontrol bacteria were developed. It was observed that 34% of the 263 isolates were able to inhibit the growth of at least one of the two fungi tested and 47% showed siderophore production in CAS medium. Analysis of nutritional similarity index demonstrated that both fungi were capable to growth in all carbon compounds assayed and that 20% of bacteria showed NOI values equal or above 0.9. Genetic diversity analysis by BOX–PCR indicated that culturable epiphytic and endophytic bacteria associated with peanut are highly diverse. The 16S rDNA sequences of the most remarkable isolates, indicated as potential biocontrol agents according to all the screenings performed and belonging to different BOX profiles showed that they were 99% identical to *Bacillus amyloliquefaciens*.

Comparisons analysis was performed with results previously obtained from a similar study done in the bacterial community isolated from healthy peanut plants from the same producing area. Results obtained suggest that presence of fungal pathogens in peanut ecosystem would be acting as a selective factor in the peanut plant associated bacterial communities. It was concluded from this study that peanut soils in Cordoba harbor bacteria with major biocontrol properties which represent a potential source of new strains that could be used as biological inoculants in agriculture.

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1. Introduction

The rhizosphere is a densely populated area where plant roots interact with soilborne microorganisms, including bacteria, fungi and invertebrates, feeding on an abundant source of organic material [30]. Negative interactions between microorganisms and plants are the most concerning in terms of sustainability and economic loses. On the other hand, it is well known that a considerable number of bacterial species are able to exert a beneficial effect upon plant growth. This group of bacteria has been termed "plant growth promoting bacteria" (PGPB) [5], and among them are strains from genera *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcalígenes*, *Arthrobacter*, *Acinetobacter*, *Flavobacterium*, etc. Bacteria that are inhabitants of plant external surfaces or internal tissues are commonly named epiphytes and endophytes, respectively [2,14,19]. Both can contribute to the health, growth and development of plants and can be found in roots, stems and leaves [35]. Many of these bacteria are able to control the growth and activity of phytopathogens by a variety of mechanisms. The production of siderophores, molecules which chelate iron or other metals, contribute to disease suppression by conferring a competitive advantage to biocontrol strains [20]. Furthermore, the production of antimicrobial substances, such as antibiotics or HCN, is an important mechanism to fight phytopathogens [3]. Indirect disease control is achieved by mechanisms modulating the plant immune response, including the induction of systemic acquired resistance [34].

To solve problems caused by crop diseases several alternatives are presented. The use of resistant cultivars is one of them but it should be limited to pathogen and plant species. On the other hand, engineered resistant plants are mainly unsuccessful because

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phytopathogens usually subdue this resistance [27]. Adding to that, use of agrochemicals is not desirable because of negative effects in environment. Then, agronomically friendly practices are required and use of biocontrol microorganisms is a technology widely studied. Their use as inoculant or control agents for agriculture improvement has been a focus of numerous researchers for a number of years [4,22,29].

Therefore, inoculation of plants with target microorganisms would be necessary to take advantage of their beneficial properties for plant yield enhancement [16]. Although plant growth promoting bacteria occur in soil, usually their numbers are not enough to compete with other bacterial strains commonly established in the rhizosphere [28]. A prerequisite for introducing these beneficial bacteria in the environment is that, in addition to plant growth promotion, they should have the ability to compete with soil microflora. Thus, it is important that native bacteria, isolated from the same ecological niche as the pathogen, are used as biological control agents [35]. The advantage of using natural soil isolates over the genetically manipulated or over those isolated from a different environmental setup, is their easier adaptation and success when inoculated into the plant rhizosphere [9]. Nevertheless, selection of bacterial species in the rhizosphere may be influenced by several factors. Both plant species and soil properties have been indicated as important steering factors [10] as well as other rhizosphere inhabitants. In this respect, the interactions with saprophytic fungi are of particular interest as they are organotrophs and may, therefore, be direct competitors for root exudates [10].

Peanut (*Arachis hypogaea* L.) is a widespread leguminous plant of great agricultural and economic significance. Argentina is one of the major peanut producers in the world, and about 80% of its production takes place in the province of Córdoba. Peanut production in this area reached 430,000 tonnes during 2009/2010 growing season. Most of the production is exported to different regions of the world, including EC countries and the USA. Diseases caused by soil-borne fungi (especially *Sclerotinia sclerotiorum*, *Sclerotinia minor*, *Sclerotium rolfsii* and *Fusarium solani*) are a limiting factor in peanut production, and one of the main causes of the reduction of the planting area in Argentina [7]. These fungi are commonly present in soils where *Arachis hypogaea* L. is growing [23,35]. In previous studies of our laboratory biocontrol mechanisms and diversity of bacteria isolated from healthy peanut plants from the producing area of Córdoba have been analyzed [35]. We hypothesized that potential biocontrol bacterial communities from healthy peanut plants differ from those growing in the rhizosphere and phyllosphere of diseased plants. To better understand the effect of phytopathogenic fungal growth on the diversity of bacterial biocontrol agents, it is necessary to compare bacterial communities inhabiting the rhizospheres of both healthy and diseased peanut plants.

The objective of the present study was to isolate and characterize endophytic and epiphytic biocontrol bacteria from peanut plants affected with root rot (caused by *F. solani)* and white mold (caused by *S. minor*).

2. Material and methods

2.1. Bacteria, fungi and culture media

Bacterial isolates were obtained as described below. Bacteria were grown in trypticase soy agar (TSA, Britania) or Luria Bertani medium (LB) [25]. The isolates were kept in 20% glycerol at −80 °C for storage and in 40% glycerol at −20 °C for maintenance. *S. minor* was maintained on potato dextrose agar (PDA) [17], while *F. solani* was grown on carnation leaf agar (CLA) [26].

2.2. Bacterial isolation procedure

Bacterial strains were isolated from peanut roots, stems and leaves of plants cultivated in three fields (hapludol soils) of the main producing area of peanut corresponding to the central and southern region of Córdoba, Argentina (latitude, 32°–34°, longitude, 63°–65°). Nine plants, with white mold symptoms from Vicuña Mackena (V. Mackena) and 7 showing root rot symptoms obtained from Coronel Moldes (five plants) and from Carnerillo (two plants) were collected at the reproductive R_6 growth phase [6]. All plants showed severe symptomatology of each fungal disease. To isolate culturable epiphytic and endophytic bacteria, peanut plants were washed in running tap water to remove soil, and the roots, stems and leaves were separated. Epiphytic bacteria were isolated from non-disinfected tissue. Three grams of roots, stems or leaves were placed in a 500 ml Erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of phosphate buffered saline (PBS: NaCl 0.14 M; KCl 0.0027 M; Na_2HPO_4 0.01 M; KH_2PO_4 0.0018 M, pH 7.4) and agitated at 150 rpm for 1 h. To isolate endophytic root, stem and leaf bacteria, epiphytes were removed by surface disinfection of plant tissues using serial washing in 70% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 70% ethanol for 30 s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% TSA and incubating the plates at 28 °C. The tissue was then macerated and treated in flasks as above. Dilutions of the contents of the flasks were plated onto 10% TSA supplemented with 50 μg ml^{-1} of cycloheximide (to control fungal growth) and the plates were incubated at 28 °C for 7 days. Morphologically different colonies were picked off each plate, suspended in 20% glycerol solution and stored at −80 °C [19]. These isolates were used to screen for potential antifungal properties. Morphology and Gram stain of isolated bacteria were examined by light microscopy.

2.3. Biocontrol traits

2.3.1. In vitro inhibitory effect on fungal growth (antibiosis)

In vitro antagonism tests were performed against *S. minor* and *F. solani*. To assess the ability of the isolates to inhibit fungal growth a single colony of each isolate growing on TSA medium was spotted onto one side of PDA plates. A 5 mm diameter plug of each fungus was centrally placed at approximately 3.5 cm from the bacterial spot. Plates were incubated at 25 °C for 3–14 days. Fungal growth inhibition was assessed regularly during 2 weeks by measuring the mycelial radial growth and compared to control growth [17]. Bacteria that inhibited or altered mycelial extension of at least 1 fungal species on PDA were considered as potential antagonists.

2.3.2. Siderophore production

The method described by Schwyn and Neilands [32] was used. Chrome azurol S agar plates were spot inoculated with bacterial cultures (10 μl of approximately 10^8 CFU/ml) and incubated at 28 °C for 2–7 days. Development of a yellow-orange halo around the colonies indicated siderophore production. *Pseudomonas* spp "BREN6" (siderophore producer), was used as reference strain [35].

2.3.3. Determination of nutritional similarity

In vitro carbon source utilization profiles were determined for each bacterium and fungus strain. Plates containing agar 1.5% were supplemented with 10 mM of one of the following compounds: D-fructose, D-glucose, D-raffinose, xylose, trehalose, L-histidine, D-melibiose, L-alanine, D-serine, L-arginine, sucrose, L-glutamic acid, L-phenylalanine, L-prolina, L-threonine, L-leucine, L-methionine and inoculated with either bacterial or fungal strains. They were incubated at 28 °C for 7 days. Growth on the sole carbon source plate

was compared with that on a negative control (agar 1.5% without carbon source). The nutritional similarities between fungi and bacteria were estimated with the NOI formula (niche overlap index) = the number of carbon sources used by both, biological control agent and pathogen/the total number of carbon sources used by the pathogen [38].

2.4. DNA preparation and BOX–PCR fingerprints analysis

DNA template from bacteria was obtained by either of two techniques: a) Colonies growing on TSA plates were collected, suspended in 300 μl of 1 M NaCl, mixed thoroughly and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was suspended in 300 μl double distilled sterile water. After the sample was mixed and centrifuged as described before, the supernatant was removed and the pellet was suspended in 150 μl of 6% (aqueous suspension) resin Chelex 100® (Bio Rad). This suspension was incubated at 56 °C for 20 min, followed by mixing and further incubation at 99 °C for 8 min [37]. b) A volume of 1.5 ml of an overnight bacterial culture in LB broth was centrifuged for 2 min at 12,000 rpm, the pellet was suspended in 750 μl of 0.1× SSC, and centrifuged again. Cells were suspended in 570 μl of 10 mM Tris–HCl/sucrose 20% (pH 8), lysozyme (2.5 mg/ml) was added and the suspension was incubated at 37 °C for 3 h. Then, 130 μl of lysis buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 1% SDS and 200 mg/ml proteinase K) were added and the solution was incubated at 37 °C for 30 min. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and after centrifugation at 12,000 rpm for 5 min, the aqueous phase was extracted. DNA was precipitated by the addition of 0.1 volume of NaAc 3 M (pH 4.8) and 2 volumes of cold ethanol. After 12 h at −20 °C, this suspension was centrifuged at 12,000 rpm for 10 min and the supernatant was discarded. The pellet was suspended in 50 μl double distilled water [11]. DNA concentration of the samples was approximately 5 ng μl^{-1}.

The sequence of BOX primer BOX-AR1 (5′-CTACGGCAAGGC GACGCTGACG-3′) used in this study has been reported by Versalovic et al. [36]. The polymerase chain reaction (PCR) was performed in 25 μl reaction mixture containing 10× PCR buffer, 50 mM $MgCl_2$, 0.2 mM each nucleotide (Promega) (2 mM DNTPs), 50 pmol/ml of primer, 1 U of *Taq* DNA polymerase (Promega) and 6 μl of template DNA solution. The reaction conditions were as follows: initial denaturation at 95 °C for 7 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 65 °C for 8 min; and a final extension step at 65 °C for 16 min. PCR-amplifications were performed in a Mastercycler gradient block (Eppendorf). The BOX amplification products in 12 μl sub-samples were separated by horizontal electrophoresis on 2% agarose gels and stained with ethidium bromide or SYBR Green®. Fingerprints profiles obtained were analyzed and dendrograms were constructed with BioNumerics Ver 3.0, Applied Maths, Belgium, employing Jaccard similarity coefficient and UPGMA [31].

The nucleotide sequences of the nearly full-length 16S rRNA genes directly obtained by Macrogen Laboratories (Korea) employing universal primers 518F (5′ CCAGCAGCCGCGGTAATACG 3′) and 800R (5′ TACCAGGGTATCTAATCC 3′) were deposited in GenBank (accession Nos. in Fig. 4). Sequence analyses of 16S rDNA were performed using the algorithm BLASTN [1] to identify similarities and to perform alignments. Phylogenetic and molecular evolutionary analyses were conducted using BioEdit [13], MEGA version 4 [33] and PHyML [12] software.

2.5. Comparison analysis

Data obtained from sections mentioned above (Sections 2.3.1–3 and 2.4.) were compared to that obtained in a previous study in which peanut native bacteria isolated from healthy plants from the same producing area of Córdoba were analyzed in their *in vitro* biocontrol properties [35]. Parameters compared were: Number of colony forming units of endophytic and epiphytic bacteria isolated from these plants, genetic diversity and percentage of bacteria showing the biocontrol traits analyzed (antibiosis against *S. minor* and *F. solani*, siderophore production and NOI index).

2.6. Statistical analysis

Data were analyzed statistically using analysis of variance comparing means with the LSD multiple mean comparison test or using the Student *t*-test. In both cases Infostat software was used. A level of $P < 0.05$ was accepted as significant.

3. Results

3.1. Isolation of culturable epiphytic and endophytic bacteria from plants affected with white mold and root rot

Abundance analysis of cultivable epiphytic bacteria and endophytes inhabiting peanut leaves, stems and roots indicated that populations were 10^5–10^6 CFU per gram dry tissue. Of the colonies obtained, 49% were epiphytes and 51% endophytes. Considering plant tissue, 41% corresponded to leaf isolates, 42% to roots and 17% to stems (Fig. 1). The largest number of isolates was obtained from the plants of the field of Coronel Moldes.

3.2. Biocontrol traits in peanut associated bacteria

A total of 263 bacteria that differed in their colony morphology, were screened for *in vitro* growth inhibitory activity against two major peanut fungal pathogens of the producing area of Córdoba. This group included 139 isolates obtained from peanut plants with white mold symptoms (sampled from Vicuña Mackena field) and 124 isolates from plants affected with brown root rot (sampled from Coronel Moldes and Carnerillo fields).

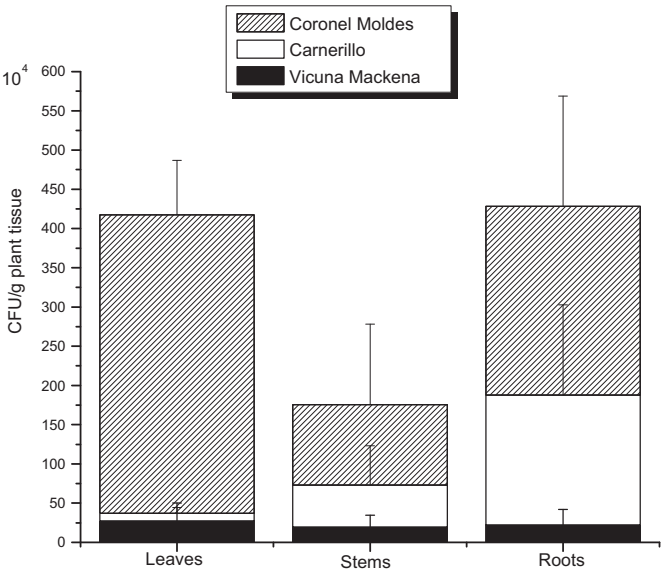

Fig. 1. Number of epiphytic and endophytic isolates from leaves, stems and roots from peanut plants affected with white mold and brown root rot obtained from three fields: Carnerillo, Vicuña Mackena and Coronel Moldes. Data represents X ± S.E., n: 3. L: leaves, S: stems, R: roots.

Table 1
Epiphytic and endophytic bacterial isolates that showed *in vitro* antibiosis against *S. minor* and/or *F. solani*, simultaneous siderophore production and their niche overlap index (NOI) values.

		Antibiosis (% of fungal growth inhibition)		Siderophore production	NOI value
		S. minor[a]	*F. solani*[a]		
Epiphytes					
From roots	AEPR 4	56.7 ± 2.0	0	+	0.7
	AEPR 8	63.7 ± 2.5	0	+	0.7
	AEPR 14	53.4 ± 1.4	0	+	0.8
	AEPR 16	49.9 ± 5.3	0	+	0.9
	AEPR 20	57.0 ± 4.7	48.4 ± 2.6	+	0.7
	AEPR 21	63.0 ± 4.9	37.5 ± 7.7	+	0.7
	AEPR 22	58.5 ± 2.4	0	+	0.8
	AEPR 28	55.5 ± 4.1	32.4 ± 7.3	+	0.8
	AEPR 32	45.5 ± 3.4	0	+	0.9
	AEPR 33	63.2 ± 8.1	0	+	0.8
	AEPR 37	56.7 ± 6.4	0	+	0.9
	AEPR 42	55.2 ± 6.0	28.5 ± 5.9	+	0.8
	AEPR 45	64.0 ± 6.0	29.2 ± 11.4	+	0.7
	AEPR 46	67.0 ± 3.0	39.7 ± 2.4	+	0.7
	CEPR 1	42.2 ± 4.1	0	+	0.8
	CEPR 2	39.9 ± 2.7	0	+	0.8
	CEPR 3	37.4 ± 2.0	0	+	0.8
	CEPR 5	35.5 ± 5.0	0	+	0.7
	CEPR 6	39.2 ± 3.0	0	+	0.8
	MEPR9	42.2 ± 5.3	0	+	0.6
	MEPR12	41.7 ± 4.4	0	−	0.8
From stems	AEPT 2	50.9 ± 4.3	0	+	0.8
	AEPT 4	45.7 ± 7.5	0	+	0.8
	AEPT 14	50.7 ± 4.5	0	+	0.7
	AEPT 15	55.5 ± 2.2	0	+	ND
	AEPT 21	49.0 ± 6.0	0	+	0.7
	AEPT 22	46.7 ± 4.5	0	+	0.9
	AEPT 23	21.0 ± 2.0	0	+	0.7
	AEPT 24	60.5 ± 4.4	0	−	0.8
	AEPT 25	64.9 ± 4.1	0	+	0.7
From leaves	AEPH 1	49.2 ± 7.8	0	−	0.6
	AEPH 2	48.0 ± 6.1	0	+	0.9
	AEPH 10	46.5 ± 8.1	0	+	0.9
	AEPH 12	48.5 ± 6.1	0	+	0.8
	AEPH 16	56.5 ± 6.6	50.2 ± 2.3	−	0.8
	AEPH 23	57.0 ± 9.1	46.9 ± 1.8	−	0.8
	AEPH 25	65.0 ± 9.0	0	−	0.8
	AEPH 26	53.5 ± 8.2	0	+	0.8
	AEPH 27	50.5 ± 6.4	0	−	0.8
	AEPH 29	56.0 ± 7.8	0	+	0.9
	CEPH 11	0	26.5 ± 5.5	+	0.8
	MEPH 13	42.9 ± 4.2	0	+	0.7
	MEPH 14	54.5 ± 3.4	0	+	0.7
	MEPH 15	44.4 ± 7.2	0	+	0.5
	MEPH 16	44.2 ± 5.1	0	+	0.7
	MEPH 29	39.5 ± 2.0	0	+	0.8
	MEPH 32	43.9 ± 2.7	0	+	0.6
Endophytes					
From roots	AENR 1	0	34.7 ± 10.5	−	0.9
	AENR 6	59.5 ± 2.8	0	−	0.6
	AENR 17	66.7 ± 2.2	0	+	0.7
	AENR 19	66.7 ± 3.3	37.7 ± 7.4	+	1
	AENR 25	65.7 ± 7.0	0	+	0.8
	AENR 26	54.7 ± 5.7	0	+	0.6
	AENR 27	56.5 ± 4.3	0	+	0.9
	AENR 29	63.7 ± 4.4	42.0 ± 4.0	−	0.8
	AENR 30	48.0 ± 5.0	23.2 ± 5.7	+	0.9
	AENR 33	62.0 ± 3.2	0	+	0.8
	AENR 34	48.0 ± 4.0	0	+	0.9
	AENR 35	45.7 ± 4.1	0	−	0.8
	MENR 13	0	34.7 ± 5.0	+	0.7
	CENR 1	38.5 ± 1.2	0	−	0.8
	CENR 5	41.5 ± 4.5	0	+	0.7
From stems	AENT 1	60.5 ± 3.1	0	+	0.7
	AENT 2	62.2 ± 1.6	0	+	0.8
	AENT 13	44.0 ± 5.0	0	+	0.9
	MENT 1	49.5 ± 7.2	0	+	0.3
	MENT 4	48.2 ± 6.1	0	−	0.5
	MENT 5	59.0 ± 3.2	0	+	0.6
	MENT 9	42.7 ± 2.5	30.2 ± 9.0	+	0.8

(*continued on next page*)

Table 1 (*continued*)

		Antibiosis (% of fungal growth inhibition)		Siderophore production	NOI value
		S. minor[a]	*F. solani*[a]		
	MENT 12	40.7 ± 1.8	34.6 ± 7.9	+	0.7
	MENT 14	42.5 ± 1.3	35.5 ± 7.4	+	0.8
	MENT 15	39.6 ± 3.0	0	+	0.5
From leaves	AENH 1	49.9 ± 7.2	0	+	0.8
	AENH 2	55.2 ± 1.8	0	–	0.7
	AENH 6	52.9 ± 3.6	47.1 ± 3.3	+	0.6
	AENH 8	49.0 ± 3.7	42.0 ± 4.5	+	0.8
	AENH 16	55.2 ± 5.8	0	+	0.9
	AENH 20	51.0 ± 5.1	0	+	0.8
	AENH 21	48.2 ± 9.3	0	+	0.7
	AENH 25	54.9 ± 4.0	0	+	0.8
	AENH 26	37.2 ± 9.5	0	+	0.9
	AENH 28	51.5 ± 7.2	0	+	0.8
	AENH 29	53.4 ± 9.5	25.5 ± 9.7	–	0.7
	AENH 31	40.7 ± 10.7	0	+	0.6
	AENH 32	49.0 ± 9.4	0	+	0.8
	AENH 33	55.2 ± 0.8	0	+	0.8
	AENH 34	51.0 ± 7.9	0	+	ND
	MENH 2	44.5 ± 3.6	0	+	0.9
	MENH 3	44.7 ± 3.8	0	+	0.6
	MENH 13	48.9 ± 1.5	0	+	ND

[a] Data are means ± S.E. Experiments were repeated 3 times, First letter of each isolate indicates the field from which it was isolated: A. Bacteria isolated from peanut plant sampled in Vicuña Mackena, M: Bacteria isolated from peanut plant sampled in Coronel Moldes, C: Bacteria isolated from peanut plant sampled in Carnerillo. ND: not determined.

When biocontrol traits were analyzed, it was observed that 34% of the 263 isolates showed antibiosis against at least one of the two fungi tested (Table 1). Seventy eight percent (78%) of them inhibited *S. minor* growth, 3% inhibited *F. solani* growth and 19% were antagonistic against both phytopathogens. Bacteria showing antibiosis against *S. minor*, were mostly isolated from roots and leaves and, the greater percentage (69%), came from peanut plants affected with white mold (Fig. 2). Most of the bacteria able to inhibit *F. solani* growth came from root tissues, and mostly (73%) from peanut plants with white mold symptoms. Siderophore production was observed in 47% of analyzed bacteria, most of them isolated from leaves and roots. Within the 263 isolates selected for *in vitro* biocontrol traits analysis, the ability of a same bacterium to inhibit growth of both fungi and also to produce siderophores was observed in 5% of isolates. Results obtained indicated that 52% of the total isolates showed at least one of these properties.

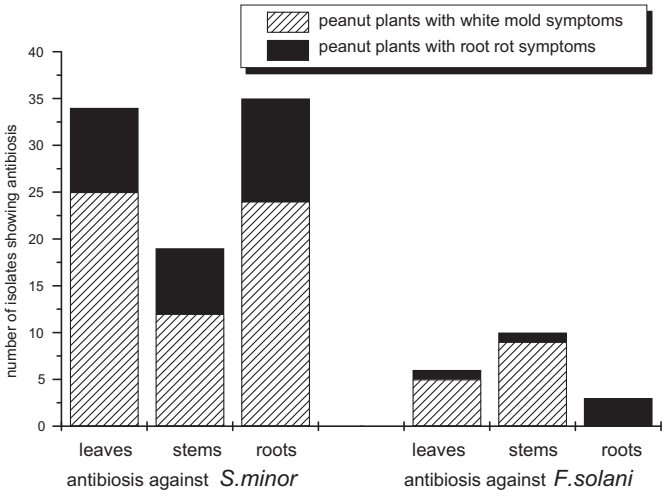

Fig. 2. Number of potential biocontrol bacteria showing antibiosis against *S. minor* and *F. solani* isolated from leaves, stems and roots from peanut plants affected with white mold and brown root rot.

Determination of nutritional similarity was analyzed in 126 isolates considering their ability to show at least one *in vitro* biocontrol property previously described. This analysis demonstrated that meanwhile both fungi were capable to growth in all carbon compounds assayed, 20% of bacteria assayed showed NOI values equal or above 0.9 corresponding mainly to leaves and roots isolates (40% and 36%, respectively).

Gram stain and bacterial morphology of the potential biocontrol bacteria indicated that 94% of them were gram positive bacilli, 3% gram positive cocci, 2% gram negative bacilli and 1% gram negative cocci (data not shown).

3.3. Genetic diversity of the biocontrol bacteria

Genetic diversity in a bacterial population composed of 23 Gram positive bacilli able to inhibit fungal growth in PDA medium, to produce siderophores in CAS media and showing a NOI $\geq$0.6 was analyzed by BOX–PCR. This analysis yielded a total of 15 different profiles, indicating that the culturable epiphytic and endophytic bacteria associated with peanut are highly diverse (Fig. 3).

3.4. Comparison analysis with biocontrol bacteria from healthy peanut plants

From this analysis, it was possible to observe that plant tissues of healthy peanut plants harbour larger populations of bacteria than those from plants infected with white mold or brown root rot pathogens as indicated by higher number of colony forming units per gram tissue (10^6–10^7 and 10^5–10^6 CFU/g plant tissue, respectively, $P < 0.05$, data not shown). No differences were observed between the two populations when estimating the number of bacteria able to inhibit the growth of *F. solani* and with the number of bacteria showing a NOI >0.9 (Fig. 5). On the other hand, percentage of bacteria able to inhibit *S. minor* growth and the one corresponding to bacteria that presented two biocontrol traits (antibiosis and siderophore production) was greater in the bacterial community isolated from diseased peanut plants (8 and 3%; 12 and 55%, respectively). Genetic diversity analysis indicated that both bacterial communities harbour a diverse group.

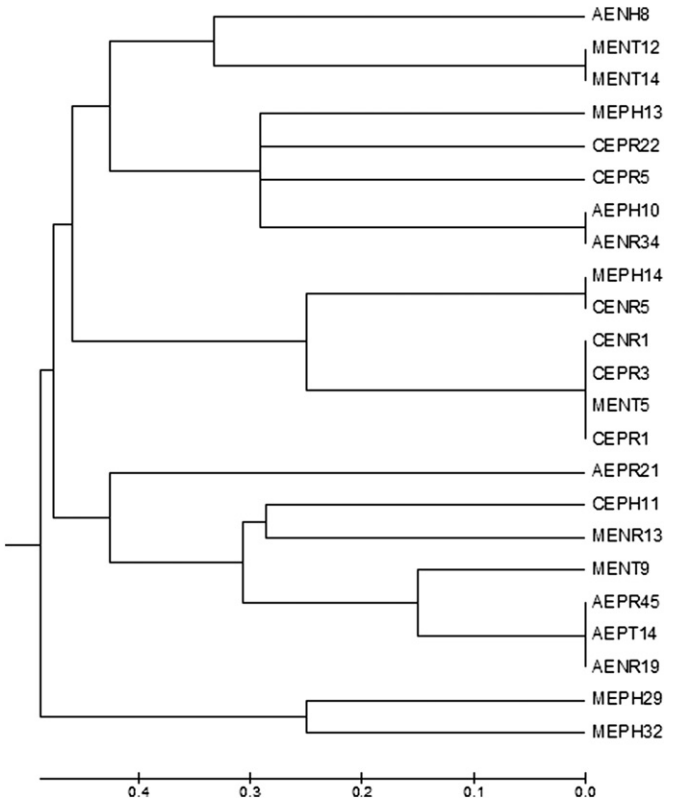

Fig. 3. Dendrogram based on the similarity matrix and through the unweighted pair-group method with arithmetic averages (UPGMA) of BOX-fingerprints of epiphytic and endophytic Gram positive bacilli isolated from peanut plants showing white mold and brown root rot symptoms.

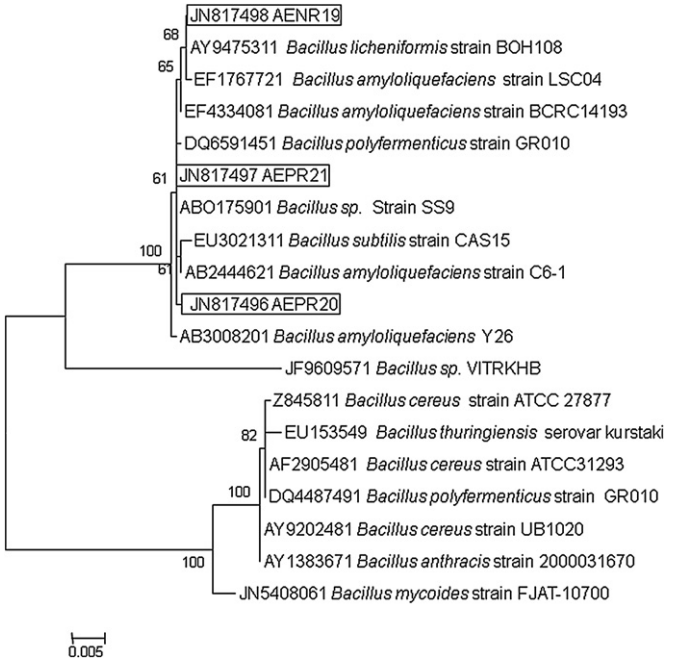

Fig. 4. Neighbour-joining phylogenetic trees based on 1470 bp 16S rRNA gene sequences of Gram-positive selected bacteria. The trees were inferred under the Tamura–Nei + G substitution model. Bootstrap values (over 50%) for 1000 replicates are shown.

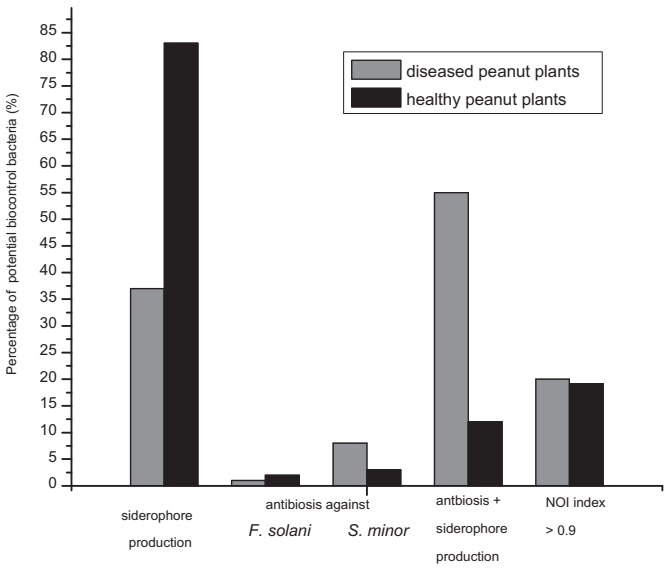

Fig. 5. Percentage of bacteria isolated from healthy and diseased peanut plants showing antibiosis against *S. minor*, antibiosis against *F. solani*, production of siderophores, simultaneous antibiosis and siderophore production, and NOI index above 0.9.

3.5. 16S rRNA gene sequencing analysis

The 16S rDNA sequences of three isolates, indicated as potential biocontrol agents according to all the screenings performed, were analyzed. Analysis of these Gram-positive bacteria showed that the three isolates were 99% identical to *Bacillus amyloliquefaciens*. Hence, phylogenetic analysis and construction of a phylogenetic tree of the Gram-positive isolates placed them in the cluster represented by *B. amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis* (Fig. 4).

4. Discussion

Isolation and characterization of endophytic and epiphytic bacteria from peanut plants affected with brown root rot (caused by *F. solani*) and white mold (caused by *S. minor*) demonstrated that diseased peanut rhizosphere harbours an important source of potential biocontrol microorganisms. *In vitro* antibiosis, siderophore production and high niche overlap index was observed mostly in leaves and roots isolates indicating that these plant tissues should be selected when potential biocontrol bacteria are being searched for. The high percentage of isolates able to inhibit *S. minor* suggested that this fungus is more sensitive than *F. solani* to the biocontrol bacteria evaluated. Nutritional similarity index is another important property to be considered for biocontrol bacteria selection. A NOI value equal or higher than 0.9 suggests a high ecological similarity between antagonistic bacteria and pathogens. This implies that both partners may compete for the same ecological niche suggesting that coexistence would not be possible. High NOI values have been used by several authors as a selective criterion when biocontrol bacteria are searched [8,18,35]. Within the most promising *in vitro* biocontrol bacteria characterized in this study, fingerprint analysis indicated a high diversity. No correlation was found between the plant tissue nor field where bacteria were recovered and genetic diversity. It is important to note that each pathogen and each biocontrol agent establishes a particular interaction and that the antagonistic effect would be the result of more than one biocontrol mechanism. When

selecting a biocontrol bacterium, integral approaches are needed to analyze all potential biocontrol mechanism that the microorganism shows. Considering the results obtained in this study, some of the isolates analyzed could be suggested as biocontrol agents. If criteria used for the selection are high *in vitro* antibiosis activity against both *F. solani* and *S. minor,* siderophore production and NOI values above 0.7, isolates AENR 19, AEPR 20 and AEPR 21 are the most promising bacteria to be used as biocontrol agents. The 16S rRNA sequence analysis of these biocontrol agents indicated that they belonged to the genera *Bacillus*. Bacteria from these genera are common inhabitants of the rhizosphere and phyllosphere, and are also well known as biocontrol agents. Interestingly, these bacteria were isolated from the same field (Vicuña Mackena) and from root, emphasizing that this tissue is a good source of biocontrol microorganisms.

A comparison analysis with the bacterial community isolated from healthy plants was proposed in order to determine if presence of phytopathogen exerts any effects on biocontrol bacteria communities. Therefore, data obtained in this study were compared with those obtained from a previous study in our laboratory on bacteria isolated from healthy peanut plants from the same producing area of Córdoba showing *in vitro* biocontrol properties [35]. Analysis of epiphytic bacteria and endophytes inhabiting peanut leaves, stems and roots from healthy and diseased plants revealed differences in the size and in the morphological and staining properties of bacterial populations. Plant tissues of healthy peanut plants harbour larger populations of bacteria than those from plants infected with white mold or brown root rot pathogens as indicated by higher number of colony forming units per gram tissue, indicating a reduction in culturable bacteria number when fungi *S. minor* and *F. solani* are present in peanut ecosystem. On the other hand, in another approach, analyzing the effect of phytopathogen presence on soil bacteria number, no differences were observed between culturable bacterial populations [10]. Contrary to that, in a study in which changes in bacterial populations associated with take-all disease of wheat were analyzed, more culturable bacteria were found in infected plants to that of non infected ones [24]. Similar findings were obtained with rhizosphere bacteria of avocado trees (*Persea americana*) infected with the pathogen *Phytophthora cinnamomi* [39]. On the other hand, when analyzing the number of gram positive or gram negative bacteria, almost all of the isolates from diseased plants were gram positive while in the healthy plants approximately half of the isolates showed positive gram reaction. It is well known that gram positive bacilli are able to form endospores which are structures that confer resistance to environmental stresses. Considering that presence of fungal pathogen is a biotic stress, the high number of gram positive bacteria could be attributed to the formation of this advantageous cell type. Genetic diversity analyzed by fingerprint analysis in the present study and by Tonelli et al. [35], indicated that potential biocontrol bacteria associated with peanut are highly diverse in both populations. Whereas most of the bacteria isolated from healthy peanut plants showed the only biocontrol ability to produce siderophores, in those endophytic and epiphytic bacteria belonging to the population from infected rhizosphere and phyllosphere it was found that a higher percentage of them showed simultaneously the three biocontrol activities analyzed. Considering that bacteria showing more than one antagonistic mechanism could be better biocontrol agents, results obtained in this study are indicating that infected peanut tissues are better sources of biocontrol bacteria than those from healthy plants.

Our results clearly demonstrated that the presence of a pathogen such as *S. minor* or *F. solani* has a significant effect on the bacterial communities. Furthermore, we showed that endophytes represent a promising source of biocontrol strains. Their use may be more successful than that of rhizosphere bacteria due to less competition with other bacteria in the apoplast [15,21]. Taking all these results together, it is possible to conclude that phytopathogens would act as a selective agent of soil, stem and leaf microflora associated to peanut, although it is not possible to rule out that other factors are also contributing to this effect, such as soil type and climate of localities from which both bacterial collections came.

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