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AGRARIAN SCIENCES

Isolation and selection of endophytic spore-forming bacteria with plant growth promoting properties isolated from *Ilex paraguariensis* St. Hil. (yerba mate)

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Abstract: Yerba mate (*Ilex paraguariensis* St. Hil.) is a species native to the subtropical regions of South America. Despite being an important crop for the region, there are few studies on the use of microorganisms to improve the growth of seedlings in the nursery stage. The objective of this study was to isolate spore-forming endophytic bacteria with plant growth promoting properties associated with yerba mate seedlings and determine their phytobeneficial effect under controlled laboratory conditions. Isolates were selected based on their sporulation capacity and evaluated for *in vitro* plant growth promoting properties (nitrogen fixation, phosphate solubilization, production of siderophores and synthesis of indolic compounds). Yerba mate seedlings were inoculated with the most promising isolates, which were identified via analyses of the sequence of their 16S rDNA gene as *Bacillus circulans* (12RS3) and *Bacillus altitudinis* (19RS3, T5S-T4). After 120 days plants showed higher root dry weight when inoculated with isolate 19RS3 and higher shoot dry weight with 19RS3 and T5S-T4. In conclusion, further studies to determine the ability of these isolates to adapt to the climatic conditions and to survive amidst the native soil microflora in yerba mate cultivated native soils, will be crucial for developing such strains as biofertilizer.

Key words: *Bacillus altitudinis, Bacillus circulans,* biofertilizer, *Ilex paraguariensis,* PGPR, yerba mate.

INTRODUCTION

Yerba mate (*Ilex paraguariensis* St. Hil.) is a plant species of south American origin. It becomes a tall tree when it grows in the wild, but it is shorter when cultivated. Its leaves are green and perennial, with slightly serrated margins (Margalot 1994, Alonso & Desmarchelier 2005). This species occurs naturally on lateritic soils ("tierra colorada") as a part of the understory layer of some forests (Alonso & Desmarchelier 2005). It requires warm (between 15 and 25°C) and moist weather (about 1300 to 2200 mm of annual precipitation per year) for optimal growth (Margalot 1994).

The leaves are consumed as an infusion macerated with either hot ("mate") or cold water ("tereré"). New yerba mate products have been introduced to the market in the last decades. Some examples are powdered yerba mate and mate flavoured baked goods and sodas, as well as toiletries with yerba mate as their main component. Potential uses are under study and

new products are being developed (Pereira et al. 2013, Huang et al. 2014).

Yerba mate is consumed mainly in the Rio de la Plata basin areas, including Paraguay, south Brazil, Uruguay and Argentina, and it is exported to several countries where it is not cultivated, mostly to Lebanon and Syria and in lesser quantities to Israel, Kuwait, China, Chile, the United States and several european countries (Alonso & Desmarchelier 2005).

The right conditions for its cultivation are found in Brazil, Paraguay, and Argentina, the latter being the main producer worldwide, with this agricultural activity being developed in the states of Misiones and Corrientes. The aerial survey commissioned by the Instituto Nacional de la Yerba Mate (INYM) in 2016 determined that the zones cultivated with yerba mate in our country represent an area of 165.200 ha, of which 144.014 ha are in the state of Misiones and the rest are in Corrientes (INYM 2019).

Misiones has yerba mate plantations with good production levels, but there is a raising concern for the increase in degraded plantation sites. The intensive monoculture agricultural system adopted for the cultivation of yerba mate, particularly in Argentina and Paraguay, along with inadequate management of the plantations, has accelerated the erosion, degradation of structure, progressive loss of organic matter and nutrients from soils, causing a reduction in its performance.

This has led to the development of management and plantation systems suitable for sustainable use over time (Montagnini et al. 2011).

Furthermore, it is necessary to find alternatives that allow the recovery of plantation sites and at the same time improve the growth of yerba mate plants starting on the greenhouse stage in accordance to current worldwide recommendations for crop management demanding a diminished use of chemical fertilizers.

This paves a way for the use of biological products for plant growth promotion as an alternative to achieve sustainable agriculture (Bich et al. 2011, Alarcón & Ferrera-Cerrato 2012). In the long term, sustainable agriculture would result in improved crop production and diminished detrimental effect on the environment as well as soil biodiversity. (Adesemoye et al. 2009, Grageda-Cabrera et al. 2012, Santos et al. 2012).

Use of native soil microorganisms as biofertilizers, biostimulants and biopesticides can improve the physiological response of the plants. Earlier studies on plant growth promoting microorganisms reported an increase in productivity of many cash crops and improved soil fertility owing to plant-microbe interactions in the rhizosphere (Kloepper et al. 1989, Bashan & Levanony 1990, Höflich et al. 1994, Bergottini et al. 2013). These organisms, widely known as PGPR (plant growth promoting rhizobacteria), are differentiated between those associated with soil particles, and those living within plant tissues, known as endophytes. The latter establish an intimate interaction with their plant hosts, living inside them during some period of their life cycle, colonizing their tissues without causing them any noticeable harm while becoming less susceptible to environmental stress, thus gaining an ecological advantage (Peña & Reyes 2007).

Plant growth promoting rhizobacteria can stimulate growth and development of crops either by biocontrol mechanisms or biofertilizer abilities (Çakmakçı & Tıngır 2001, Glick 2012). Some strains can also inhibit growth of phytopathogenic fungi (biological control of fungal plant pathogens), which makes them useful for biocontrol purposes (Leelasuphakul et al. 2008, Corrales Ramírez et al. 2012).

The capacity to fix atmospheric nitrogen into compounds that can be directly used by the plants is one key property of plant growth promoting bacteria. Plants use nitrogen to synthesize aminoacids, proteins, nucleotides and other essential cellular components. Even though N_2 is the main component of atmospheric air (constitutes about 78 % of it), plants are not capable of using molecular nitrogen (Curtis & Barnes 2000). Greatest nitrogen loss from the agricultural soil occurs as a consequence of harvesting procedures leading to a constant depletion in soil nitrogen content. Addition of different nitrogen sources as fertilizers showed a higher number of leaves, higher leaf area and a higher biomass production in yerba mate plants when compared to non-fertilized controls (Gaiad et al. 2006).

Phosphate is an essential mineral nutrient used by plants to synthesize high energy compounds (ATP and ADP), phospholipids, nucleic acids, and several essential coenzymes. It must be in a soluble form ($H_2PO_4^-$, HPO_4^{2-}) so that it can be absorbed through the roots, which means not all of the phosphate present in the soil is available for plants (Curtis & Barnes 2000). Even though the application of chemical fertilizers provides phosphate, a considerable part of this element is readily immobilized after application, becoming unavailable for plant uptake (Glick 2012). Some microorganisms can solubilize phosphate by releasing organic acids in the rhizosphere (Khan et al. 2009). This is an advantage because phosphate reserves present in the soils can be used, diminishing the need for the application of chemical fertilizers (Beltrán Pineda 2014, Glick 2012). Unfortunately, the results of the commercial application of phosphate-solubilizing PGPR are variable and the best results were obtained when accompanied by another property such as nitrogen fixation (Glick 2012).

Iron has important functions on plants at a cellular level as a component of cytochromes and chloroplasts. Plants absorb it from the soil as Fe^{+2} and Fe^{+3}, as well as combined with siderophores (Curtis & Barnes 2000). These are compounds that bind iron and are also synthesized by several microorganisms. It is reported that they can stimulate plant growth by two mechanisms: by facilitating iron acquisition by the plant, and by making this element unavailable to other kinds of microorganisms such as phytopathogens (Sharma & Johri 2003).

Several kinds of plant growth hormones have been described. Auxins are the most important, as they promote plant growth through many mechanisms including the formation of adventitious roots, the elongation of the stem and roots and the stimulation of fruit development (Curtis & Barnes 2000). The main auxin found in plants is indole-3-acetic acid (IAA). Different IAA concentrations affect plant physiology in different ways, and responses vary from one plant to another, the tissues involved and the developmental stage of the plant (Glick 2012). Bacterial auxins can increase hormone levels in plants from suboptimal to optimal and stimulate root growth or cause supraoptimal levels and thus inhibit root growth (Olanrewaju et al. 2017).

Some examples of PGPR are bacteria of the genera *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia* and *Bacillus*. Along with the benefits that PGPR can provide to plants, some of them can produce spores (Basualdo et al. 2006), which are resistance structures that allow them to resist adverse conditions, extending their biofertilizing effect over time. Such is the case of bacteria from genus *Bacillus*, which are resistant to environmental stress conditions and linger in the soil under adverse cultivation conditions like acidic soils and high temperatures, which constitutes an advantage

for their use as biocontrol or biofertilizer agents (Polyanskaya et al. 2002, Ochoa-Velasco et al. 2016).

Growth promoting capacities of some microorganisms have already been used in agricultural practices by inoculating seeds or plants of multiple crops. Application of endophytic and rhizospheric bacteria showing nitrogen fixation, phosphate solubilization, and IAA synthesis caused an increased dry weight of inoculated lettuce (*Lactuca sativa*) plants against non-inoculated controls (Peña & Reyes 2007). Also, endophytic bacteria inoculated to tomato (*Solanum lycopersicum*) plants increased the nutritional efficiency of the plants and incremented production (Baston Barretti et al. 2008). There are many studies on the effect of fertilization on plant growth in yerba mate in its early stages, but they focus on chemical products. The addition of nitrogen is reported to cause an increase in plant biomass (Gaiad et al. 2006), phosphorus causes increases in total height, dry matter of the aboveground part, and dry matter of the root (Santin et al. 2008, Barbosa et al. 2008). However, there are no reported works assaying the effect of PGP microorganisms on yerba mate, except for those of our group (Bergottini et al. 2015a).

Keeping in mind the utility and benefits of biofertilizers in enhancing crop yield and in improving soil health, the objective of the study was to isolate spore-forming endophytic bacteria with plant growth promoting properties associated with *Ilex paraguariensis* seedlings. In addition, the work intended to determine their effect under controlled laboratory conditions, seeking to obtain strains suitable to be used as biofertilizers.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Thirty *Ilex paraguariensis* St. Hil. seedlings acquired from a commercial greenhouse, which were grown without fertilizers, were used. They were not genetically modified and presented good phytosanitary aspect.

Sample processing consisted of harvesting, washing and separating each seedling into three sections (root, stem and leaves), which were superficially disinfected by submerging them in a 70 % ethyl alcohol solution for 5 minutes and then in a 1 % sodium hypochlorite solution for 20 minutes. A final rinse was performed with sterile distilled water, repeating the process five times or until the water was clear (modified from Pérez et al. 2016). Each disinfected part was macerated separetely using a mortar and pestle in sterile conditions with 9 mL of sterile saline solution (0.9 % sodium chloride in distilled water). The suspensions were filtered through sterile medical gauze to proceed with the selection of spore-forming bacteria. Inoculation in nutrient agar plates of the water from the last rinse was used to confirm superficial disinfection.

Selection of spore-forming bacteria

A 1.5 mL aliquot of the filtered suspension was exposed to progressive heating (heat shock) which allowed the selection of microorganisms in spore form. The heat shock scheme consisted in heating the sample up to 50 °C for 20 minutes, then to 70 °C for 20 minutes, and finally to 80 °C for 15 minutes. A 100 µL aliquot of each heated sample was inoculated in nutrient agar plates using a triangle shaped glass cell spreader (Drigalski spatula) and the plates were incubated at 30 °C for 48 hs. Colonies that were macroscopically different were selected from each plate. Schaeffer & Fulton stain was used to

microscopically confirm the presence of spores (Schaeffer & Fulton 1933).

In Vitro plant growtn promotion tests

Atmosferic nitrogen fixation

A nitrogen-free medium, which only allows the development of microorganisms that can utilize atmospheric nitrogen as their sole nitrogen source, was used. Isolates were streak inoculated in agar plates containing a modification of ATCC medium 240 (Azotobacter medium) consisting of 0.05 g K_2HPO_4, 0.15 g KH_2PO_4, 0.02 g CaCl, 0.20 g $MgSO_4$ $7H_20$, 0.002 g Na_2MoO_4, 0.01 g $FeCl_3$ $2H_2O$, 1.0 g $CaCO_3$, 15 g agar, 20.0 g sucrose per litre, and incubated in aerobiosis at 30 °C for 48 hs. Growth of the microorganism was considered as positive test, and the lack of it was regarded as a negative result.

Phosphate solubilization

National Botanical Research Institute Phosphate (NBRIP) medium was used (Nautiyal 1999). It contains insoluble phosphate salts that make it turbid; when these are solubilized by microorganisms the zones around the colony become clear. Bacterial isolates were spot inoculated on the surface of agar plates containing NBRIP medium and incubated in aerobiosis at 30 °C for 7 days. A clear halo around colonies indicated a positive result.

Synthesis of siderophores

Bacterial capacity to synthesize and secrete siderophores when grown under Fe^{3+} limiting conditions was evaluated in Chrome azurol S (CAS, Biopak Argentina) agar according to Schwyn & Neilands (1987). When the Fe^{+3} is removed from CAS due to siderophores high affinity for cations, the colour of CAS turns from blue to orange. Isolates were spot inoculated on the surface of CAS agar plates and incubated at 30 °C for 10 days. Orange halo around the colonies indicated a positive test.

Indolic compounds production capability

Isolates that gave a positive result in at least two of the previous tests were assayed for indolic compounds production (like indole acetic acid). A colorimetric method was used (adapted from Gomes et al. 2017). Each tested isolate was inoculated in a tube containing nutrient broth and incubated in aerobiosis at 30 °C for 24 h. Optical density of each bacterial suspension was measured using a spectrophotometer and was then adjusted to 0.5 (600 nm) with sterile nutrient broth. An amount of 350 µL of each adjusted suspension was inoculated in 35 mL of nutrient broth enriched with 10 ppm of L-tryptophan (a precursor in the synthesis of auxins) and incubated at 30 °C. After 24, 48, 72 and 96 h of incubation, 1 mL aliquots of each culture were centrifuged at 6000 g for 10 min. Aliquots of 500 µL of each supernatant were mixed with 500 µL of Salkowski reagent (ferric chloride in concentrated sulphuric acid) and the samples were incubated in the dark at 30 °C for 30 min (similarly to the S1/1 method used by Glickmann & Dessaux 1995). Absorbance values were measured in a spectrophotometer (UV-330 Spectrophotometer, Mapada Instruments, China) at 535 nm.

Positive and negative controls

For the nitrogen fixation, phosphate solubilization and indolic compounds synthesis tests, a collection strain from the Instituto de Biotecnología Misiones "Dra. María Ebe Reca" (InBioMis), characterized by fenotypical and genotypical methods as *Kosakonia radicincitans* (Bergottini et al. 2015b) was used as positive control. For the siderophores synthesis test, a strain of *Pseudomonas fluorescens* strain CHA0 (Tarnawski et al. 2006) was used as positive

control. *Escherichia coli* ATCC 35218 was used as negative control for all tests.

Strain selection for indentification and bioinoculation at laboratory level

Three isolates that showed better growth and that remained viable, with stronger positive results (larger halos) for the previous tests and also the ability to synthesize indole compounds were selected for molecular identification and for plant growth promotion assay under controlled laboratory conditions.

Molecular identification of endophytic spore-forming bacteria (of selected strains)

DNA extraction

Three isolates designated as 12RS3, T5S-T4 and 19RS3 with the best performance for *in vitro* plant growth promotion tests were selected. The isolates were cultivated in nutrient broth (Britania Lab. SA) at 30° for 24 h. DNA extraction procedures were done using Sambrook work protocol modified by Cariaga Martinez & Zapata (2007). DNA was resuspended in 20 µL of sterile distilled DNAse-free water (BioPack®). The extracted DNA was qualitatively evaluated in agarose gel (1 % w/v) electrophoresis stained with a solution of Gel Red (10,000 x) and quantitatively evaluated spectrophotometrically (Sambrook et al. 1989).

Amplification of 16S rDNA region

For the molecular identification and phylogeny analysis, the 16S rDNA region was sequenced. Ribosomal DNA fragments (16S) of the three selected isolates were amplified and sequenced. For the amplification reaction primers Eub9_27 and were used (Liesack et al. 1991). The amplification reactions were prepared in a final volume of 60 µL, containing 1X PCR Buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 3 mM $MgCl_2$, 100 mM of dNTP mix (InBio, Argentina), 10 pmol of each of the amplification primers, 0.5 U of Taq polymerase (InBio, Argentina), and approximately 10 ng $\mu L^{-1}$of genomic DNA. The amplification protocol consisted of an initial denaturation at 94°C for 5 min, followed by 35 PCR amplification cycles of 94°C for 30 s, 50°C for 60 s and 72°C for 60 s. A final extension step of 72°C for 5 min was included. The amplified fragment was stained and resolved on agarose gel (2 % w/v) electrophoresis. Both strands of PCR products were sequenced by Macrogen Korea for further phylogenetic studies.

Sequence analyses

The sequences sent by Macrogen were analyzed separately, manually modified or trimmed when necessary, aligned, and the consensus sequence constructed using Geneious 11.0.3 (https://www.geneious.com). The 16S rDNA sequences generated in this study for 12RS3, T5S-T4 and 19RS3 isolates were deposited in GenBank under accession numbers: MH883314, MH883235 and MH883312 respectively.

Obtained sequences were compared with the deposited information in the National Center for Biotechnology Information database (NCBI, www.ncbi.nlm.nih.gov) with BLAST Search tool. Approximately 98 accessions of the 16S rDNA sequences were selected and retrieved. These sequences represented most of the species within the *Bacillus* genus with available molecular data. The DNA sequences were aligned using the Clustal W program (Thompson et al. 1997). The selected sequences with the highest percentage of similarity were analyzed by phylogenetic methods based on one distance-based method (Neighbor joining - NJ) and two cladistic methods (Maximum Parsimony - MP and Maximum Likelihood - ML). For the phylogenetic tree evaluation, the Bootstrap analyses were conducted based on

1,000 replicates. The MEGA 6.0 (Tamura & Nei 1993) package was used for the analyses. In all analyses, 16S rDNA sequences of *Salmonella enterica* and *Escherichia coli* were used as an out-group.

Plant growth promotion assays on *I. paraguariensis* seedlings under controlled laboratory conditions

The experimental design comprised of 5 treatments: T5S-T4, 12RS3, 19RS3, a positive control consisting of YD4 (*K. radicincitans*) and distilled water as negative control. Six month old yerba mate seedlings grown in 160 mL plastic conical tubes were used. Thirty seedlings grown in commercial greenhouse substrate were inoculated per treatment with 5 mL of a suspension of $1.5x10^8$ CFU mL^1 each. The seedlings were kept under controlled laboratory conditions in a growth chamber at 25 °C, employing an 8 h photoperiod with a photon flux density of 400 µmol m^2 s^1 and 55-60 % of relative humidity. After 120 days, the height of each seedling was recorded, and seedlings were separated into roots and shoot (steam and leaves). Roots were washed to eliminate the substrate, and fresh weight was measured for roots and shoot. Each part was then oven dried at 45 °C until constant weight to determine dry weight. ANOVA tests and least significant difference (LSD) tests were used for statistical analysis using Statgraphics version 15.2.06.

RESULTS

Isolation and selection of endophytic spore-forming bacteria

Forty-seven endophytic spore-forming bacteria isolates were obtained from roots, stems and leaves of *I. paraguariensis* St. Hil. seedlings. All the isolates were purified, subcultured and stored for the *in vitro* tests.

In vitro plant growth promoting tests

Thirty-eight isolates fixed atmospheric nitrogen, 14 solubilized phosphate and 37 synthesized siderophores (Table I and Figure 1). Thirty-three isolates were positive for at least two of these assays and were tested for their ability to synthesize indolic compounds. The highest values for this parameter were recorded at 72 h and are presented in Table I.

Molecular identification of endophytic spore-forming bacteria

Using the methods described previously it was possible to obtain DNA of good quality and a PCR band of approximately 1700 bp of the 16S rDNA gene for the three selected isolates amplified with the primers Eub9_27 and Eub1542.

The consensus sequence of each isolate when compared to deposited sequences in NCBI database, showed over 98 % identity with the members of *Bacillus* genus. These high identity values (with E value 0) allowed us to assign 12RS3 isolate as *Bacillus circulans*, and the isolate T5S-T4 and 19RS3 as *Bacillus altitudinis*.

The sequences were analyzed separately by NJ, MP and ML analyses, and the resulting trees were compared. No divergences were detected between phylogenies, thus indicating that the datasets could be combined. NJ, MP and ML analyses were useful for discriminating the selected isolates at the species level (Figure 2).

All *Bacillus* sequences made sole group with high bootstrap value (99 %). The 12RS3 sequence was in a monophyletic group (bootstrap support of 91 %) with all *B. circulans* sequences. The T5S-T4 and 19RS3 sequences were in a monophyletic group (bootstrap support of 91 %) with all *B. altitudinis* and *B. aerophilus* sequences. Liu et al. (2015) reported that *B. aerophilus* and *B. altitudinis* are the same species. So, the phylogenetic analyses confirmed the taxonomic identity of the three isolates. Particularly, the *B.*

Table I. Results for the atmospheric nitrogen fixation, phosphate solubilization, synthesis of siderophores (qualitative results) for all the spore endophytic forming bacteria isolates and ability to synthesize indolic compounds (expressed as equivalent of indole acetic acid in μg μL^{-1}) tests for isolates that were positive in at least two of the other tests. (+: positive test, small halo. ++: positive test, medium halo. +++: positive test, big halo. -: negative test. NT: not tested).

Isolate	Nitrogen fixation	Phosphate solubilization	Synthesis of siderophores	Synthesis of indolic compounds (72 h)
1RS-1	-	-	+	NT
1TS-1	+	+	+	0.34
3RS-1	+	++	-	0.91
5RS-1	-	-	+	NT
5RS-2	-	-	+	NT
5RS-3	+	-	+	3.99
5RS-4	+	+	+	3.47
5RS-5	-	-	-	NT
8RS3	+	+	++	1.29
8RS4	++	+	++	0.96
8RS5	+	-	++	1.07
8RS6	+	+	++	1.02
10RS1	++	-	+	1.00
12RS1	++	-	-	NT
12RS2	+	-	+	1.00
12RS3	+	+++	+++	0.79
13TS1	+	-	-	NT
14RS2	+	-	+	0.86
15RS1	++	-	++	1.02
15TS1	+	-	-	NT
16RS1	++	-	+	4.51
16HS1	++	++	+	1.32
16HS2	+	++	+	1.13
16HS3	++	-	+	1.19
17TS1	-	-	+	NT
17TS2	-	-	+	NT
17TS3	-	-	-	NT
17TS4	-	-	-	NT
19RS1	++	-	+	0.93
19RS2	+	-	+	1.00
19RS3	++	+	+	0.87
20RS1	++	-	+	1.13
20TS1	+	-	+	1.07
21RS1	++	-	+	0.88
25HS1	++	+	+	4.41
29RS1	+	+	+	1.27

T5S-T3	+	-	-	NT
T5S-T4	+	+	+	1.01
T5S-T1	+	-	-	NT
T5S-H1	-	++	+	4.44
T5S-T2	+	-	-	NT
T5T-H1	+	-	+	4.28
T1T-R2	+	+	+	4.30
T1T-R3	+	+	+	4.60
T5T-H2	+	-	+	4.35
T5T-H3	+	-	+	4.44
T5T-H4	+	-	+	4.82
Total: 47	**38**	**16**	**37**	**33**

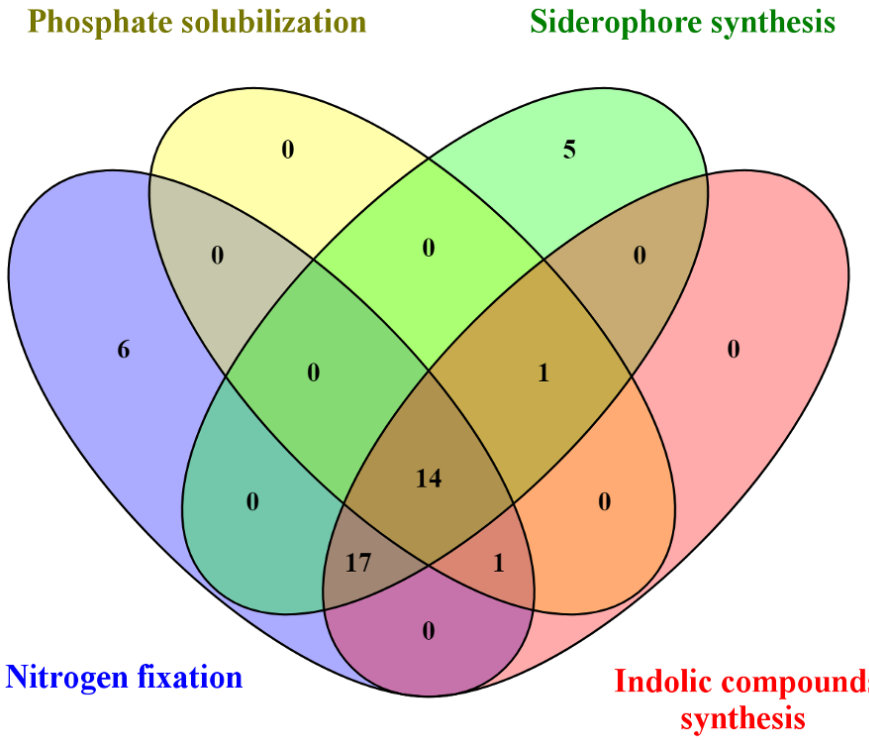

Figure 1. Venn diagram representing the number of isolates positive for each test. For the nitrogen fixation, phosphate solubilization and siderophore synthesis, a total of 38 isolates were evaluated. For the indolic compounds synthesis test 33 isolates (that were positive of at least two of the former tests) were evaluated. The values indicate the counts of the positive isolates for each test or test combination. The diagram was generated with Venny 2.1 (Oliveros 2015).

altitudinis and *B. safensis* clades were grouped as a unique cluster.

Evaluation of the biofertilizing effect of the five treatments (T5S-T4, 12RS3, 19RS3, positive and negative controls) on *I. paraguariensis* seedlings under growth chamber conditions

Analysis of variance for roots and aerial parts dry weight showed statistical differences among the means for the five treatments at a confidence level of 95.0 %. The p values were 0.0012 and 0.0294, respectively.

Figure 3 shows the results for the LSD test of root dry weight. The treatment 19RS3 showed the highest root dry weight (mean value of 0.5082 g), with statistical differences with both controls, followed by T5S-T4 treatment (mean value of 0.2666 g). The 19RS3 treatment showed an increase of 352.92 % with respect to the negative control.

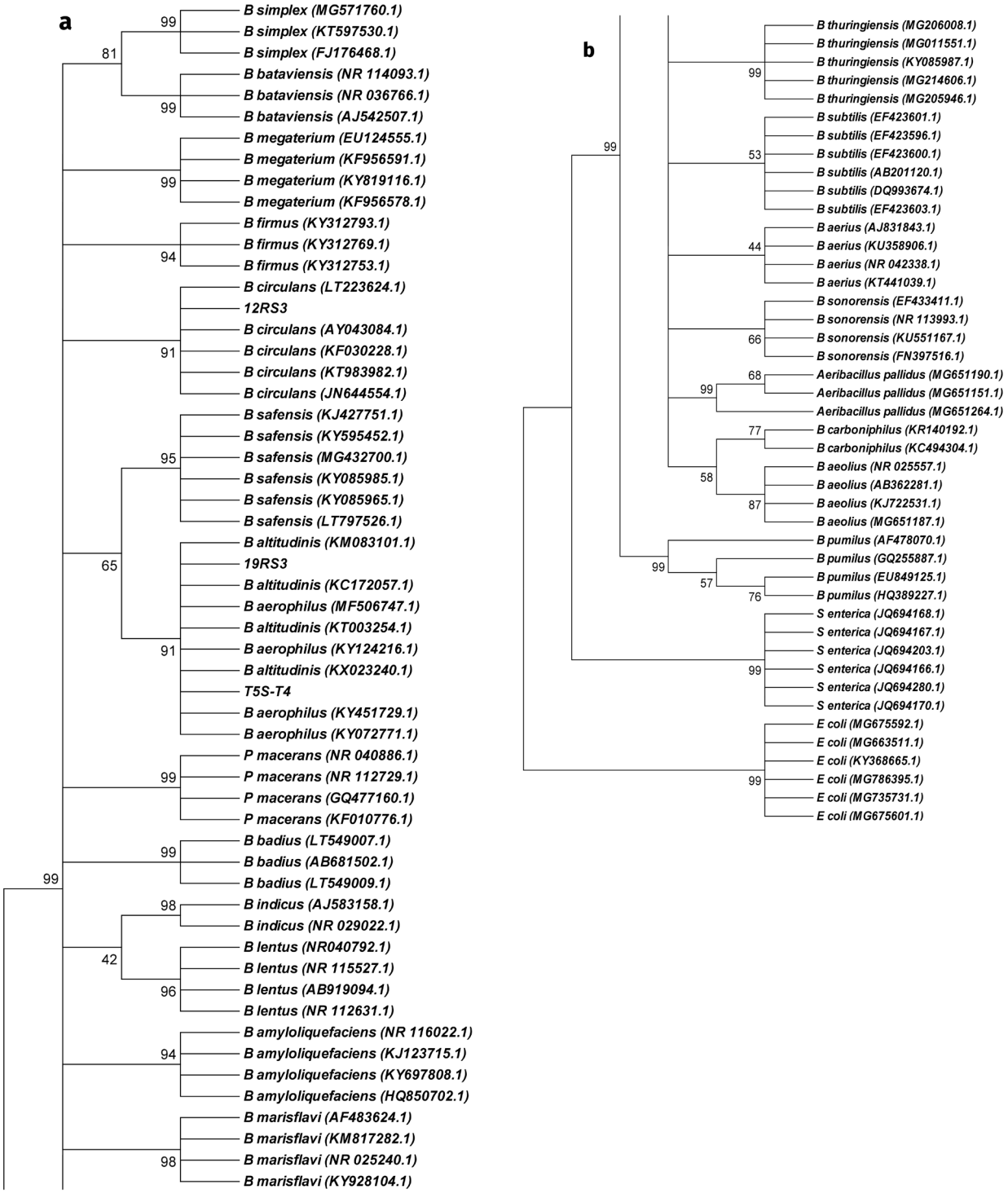

Figure 2. Phylogenetic tree of *Bacillus circulans* (12RS3), *Bacillus altitudinis* (T5S-T4 and 19RS3) based on Neighbor Joining method of 16S rRNA gene. Bootstrap test with 1000 replications. Bootstrap values (%) are given at the nodes.

Figure 4 shows the results for the multiple comparison tests of aerial part dry weight. Two treatments, 19RS3 and T5S-T4, with means of 0.9783 and 0.9791 g, respectively, showed no statistical differences between them. Nevertheless, they did show statistical differences with respect to both controls. The means for the negative and positive control were 0.6125 and 0.5379 g, respectively.

Likewise, 19RS3 and T5S-T4, compared with the negative control, showed an increase in aerial parts dry weight of 59.72 and 59.85 %, correspondingly.

The means of the height values showed no differences between the five treatments at a 95 % confidence level (p = 0.1226).

DISCUSSION

Currently, anthropogenic activity, such as the use of chemical fertilizers, is associated with the destruction of the environment. More ecological strategies are required to increase nutrient availability in agricultural lands. This feat can be achieved using endophytic and free-living rhizobacteria with plant growth properties that can be implemented in important crops for South American regions such as yerba mate.

The first stage of our research was the isolation of endophytic spore-forming bacteria from tissues of yerba mate seedlings. Bacteria were then subjected to *in vitro* plant growth promotion tests to determine their capacity to fixate nitrogen, solubilize phosphate and synthesize indolic compounds and siderophores.

The biological fixation of nitrogen is a property that promotes the growth of plants and is highly reported and investigated in crops such as rice, sugarcane, and tomato (Naher et al. 2018, Canuto de Lima et al. 2003, Botta et al. 2013).

To the best of our knowledge, there are no studies which report the influence of nitrogen-fixing spore-forming bacteria on the growth of yerba mate seedlings. Pérez et al. (2016) isolated endophytic nitrogen-fixing bacteria associated with *Ilex paraguariensis* and identified the genera *Micrococcus*, *Bacillus*, *Methylobacterium*, *Curtobacterium*, *Paenibacillus*, *Brevundimonas*, *Roseomonas* and *Mycobacterium.* However, the authors did not evaluate the effect *in vivo* of endophytic bacteria with this ability in yerba mate.

In this study, out of the 47 spore-forming isolates tested, 38 isolates were able to fix nitrogen in nitrogen-free medium. Our results resemble those of Rariz et al. (2013) who recovered 20 nitrogen-fixing out of 80 endophytic bacteria isolated from the root and aerial tissues of rice. However, the diazotrophic activity in their work was verified by acetylene reduction assay (ARA) and amplification of the *nifH* gene. Other authors, such as Majeed et al. (2015), isolated 7 nitrogen-fixing out of 9 rhizospheric and endophytic bacteria from wheat by ARA and, like us, verified their benefits in the growth of plants. During the last years, the researchers identified a great diversity of nitrogen-fixing bacteria that establish a symbiotic relationship with specific crops (Olanrewaju et al. 2017, Rosenblueth et al. 2018). Considering this, an advantage of nitrogen fixers over chemical N fertilization is that the former have regulatory mechanisms that activate the nitrogen-fixing process only when the levels of this nutrient are low (Halbleib & Ludden 2000, Santin et al. 2008).

Phosphate solubilization is another plant growth promoting property reported for many authors in microorganisms isolated from different crops including yerba mate. During the last decade, the interest in using microorganisms capable of solubilizing organic and mineral phosphates through processes that include

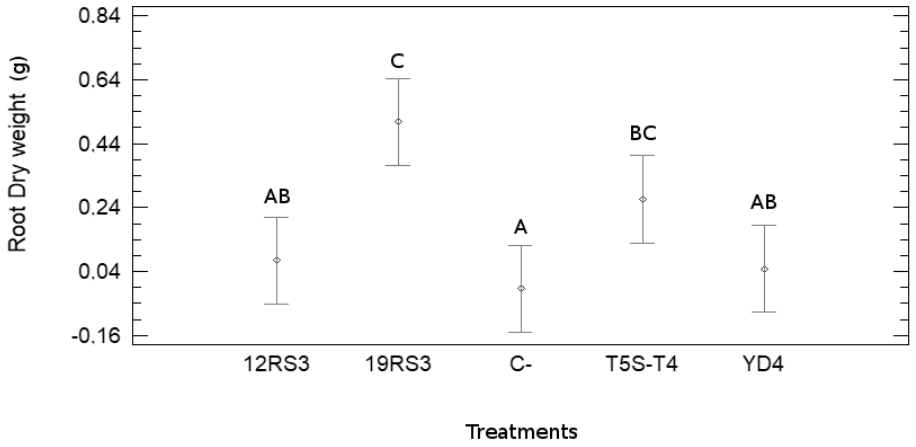

Figure 3. Least significant difference (LSD) test for the root dry weight (g) at 120 days after inoculation with *Bacillus circulans* (12RS3), *Bacillus altitudinis* (T5S-T4 and 19RS3), positive control (*Kosakonia radicincitans* -YD4) and negative control (distilled water – C-) at 95 % confidence level. Letters have been added to indicate LSD groups.

chelation, acidification and exchange reactions was increasing (Harris et al. 2005). We found that out of 47 studied isolates, 16 were capable of solubilizing phosphate. While we worked with endophytic bacteria, Becerra et al. (2011) isolated microorganisms from soils planted with cape gooseberry (*Physalis peruviana* L.) and recovered 5 isolates that solubilize phosphate with the SMRS1 medium (Sundara & Sinha 1963). Unlike the present work, these authors did not perform *in vivo* studies.

Tejera-Hernández et al. (2013) isolated microorganisms from soil, roots and stem of rice. Like us, they focused on bacteria from the genus *Bacillus* and determined phosphate solubilization using NBRIP medium. Out of 58 isolates, they found that 19 presented this growth promotion property. Like them, other authors like Collavino et al. (2010) reported similar a rate of phosphate-solubilizing bacteria. They found that out of 2700 bacterial colonies isolated from soil, rhizosphere, and soil free from roots of yerba mate, only 518 tested positive results for *in vitro* phosphate solubilization. Moreover, the working group of Collavino using a methodology like ours in yerba mate found 17 endophytic bacteria capable of growing in the NBRIP medium indicating phosphate solubilization capacity. They were also able to identify bacteria from the genus *Bacillus*. However, the isolates were from leaves and stem from yerba mate (Pérez et al. 2016).

A review on Plant Growth-Promoting Bacteria indicates that better results are obtained with phosphate-solubilizing bacteria when this property is accompanied by others (e.g., nitrogen fixation) (Glick 2012). Fortunately, our isolates also can fix nitrogen, produce IAA and synthesize siderophores.

Indolic compounds production has been studied in the rhizospheric bacteria of crops like banana, corn, grasses and cotton (Lara et al. 2011). In this study we detected this property in 33 of the studied isolates. Our results can be compared with those of Gomes et al. (2017) who studied endophytes isolated from banana tree roots and determined that out of 40 isolates studied, 16 were positive for indole production. As like us, they used Salkowski's reagent to screen indole production by endophytic bacteria. Subsequently, they quantified all positive strains using High Performance Liquid Chromatography (HPLC) and found that IAA was the main indole produced. The highest concentration obtained was 1.95 μg mL^{-1} for a *Bacillus* sp. strain, and the lowest concentration was observed for a *Bacillus pumilus* with an average of 0.30 μg mL^{-1}, which is consistent with the values that we obtained of 0.34 and 4.60 mg μL^{-1} using Salkowski's reagent.

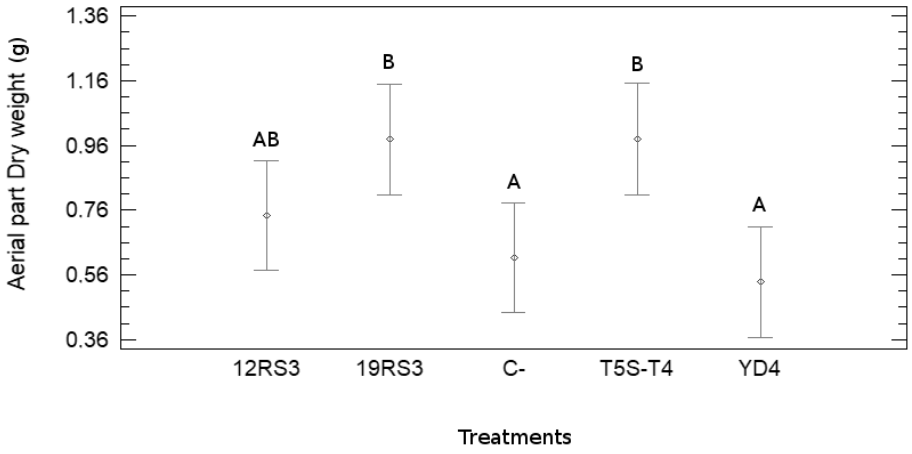

Figure 4. Least significant difference (LSD) test for the shoot dry weight (g) at 120 days after inoculation with *Bacillus circulans* (12RS3), *Bacillus altitudinis* (T5S-T4 and 19RS3), positive control (*Kosakonia radicincitans* - YD4) and negative control (distilled water – C-) at 95 % confidence level. Letters have been added to indicate LSD groups.

In the same way, López Ortega et al. (2013) evaluated 9 diazotrophic bacterial strains isolated from different soils, crops and climatic conditions and established that all tested strains synthesized indolic compounds from tryptophan. Although these strains were not isolated from maize, they did show growth promotion of said crop. In contrast to our results, these authors found higher concentrations of IAA production of the order of 63.03 µg mL^{-1}.

Importantly, there are no previous investigations on yerba mate seedlings that use isolates capable of producing IAA, except those of our research group. In these researches twelve bacterial strains isolated from yerba mate rhizosphere were assigned to six genera: *Kosakonia*, *Pseudomonas*, *Acinetobacter*, *Sphingobium*, *Rhizobium*, and *Ensifer*, based on their partial 16S rRNA gene sequence. All were able to synthesize IAA, values between 3.7 and 59.2 µg mL^{-1}/OD600. These isolates also presented the other properties of plant growth promotion. As now, three isolates were selected to advance with the *in vivo* assays (Bergottini et al. 2015a).

Earlier studies performed in different crops reported the ability of several microorganisms to synthesize siderophores (Hernández et al. 2004, Machuca & Milagres 2003, Radzki et al. 2013), some just like us described as endophytes (Teixeira Lacava et al. 2008). We found that 37 of the isolates studied in the present study showed this property.

Our work is comparable to that of Sheeba et al. (2017), who isolated bacteria from different sources like oil-contaminated soil, sea and drinking water and paddy fields. Out of 32 bacterial isolates, 14 of them produced siderophores using CAS medium.

Pandey et al. (2018) working with soil samples found that 20 isolates exhibited good siderophore production in CAS medium out of 120 isolates. Similarly to our research, they selected endospore-forming bacteria, but contrary to us, they worked with non-rhizospheric soil treated at 80 °C.

Compared to those two researches, we obtained a much higher rate of siderophore producing bacteria, since we were able to identify 37 out of 47 isolates that exhibited this property. In both cases, the authors detected other PGP properties in some of those isolates just like us, incluing phosphate solubilization and indole production.

In the second stage, once the plant growth promoting properties of the spore-forming endophytic isolates were detected *in vitro*, three isolates (12RS3, 19RS3, T5S-T4) that maintained their viability and presented all the properties

studied were selected to continue the molecular identification and *in vivo* tests.

The three endophytic spore-forming isolates were molecularly identified using the 16S rRNA gene sequencing as *Bacillus circulans* (12RS3), and as *Bacillus altitudinis* (T5S-T4 and 19RS3).

One of the advantages of the use of 16S rRNA gene sequencing is to identify a bacterial genus and species that would otherwise involve a complex and extensive biochemical identification procedure (Kawai et al. 2017). The 16S rRNA gene sequences have been by far the most common housekeeping genetic marker used. Drancourt et al. (2000) made several recommendations concerning proposed criteria for 16S rRNA gene sequencing as a reference method for bacterial identification. He established a minimum of 500 to 525 bp sequencing and a 99.5 % sequence similarity for 16S rRNA gene sequences. This work supported Drancourt's guidelines for including full 16S rRNA gene sequences whenever possible and, for groups such as *Bacillus* species that absolutely require it for accurate species identifications (Janda & Abbott 2007).

Although 16S rRNA gene sequencing is highly useful for bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genus (Bosshard et al. 2006, Mignard & Flandrois 2006). DNA relatedness studies are necessary to provide absolute resolution to these taxonomic problems (Janda & Abbott 2007). For this reason, we proposed to continue the molecular and genomic analysis of the selected isolates.

The third stage of our study advanced with the evaluation of the PGP properties in yerba mate seedlings *in vivo* under controlled laboratory conditions.

In this study, we found an increase of 352.92 % in root dry weight with respect to the negative control with 19RS3. We obtained increases of 59.72 and 59.85 % in aerial part dry weight for yerba mate seedlings inoculated with isolates 19RS3 and T5S-T4, respectively, versus the negative control.

In contrast, we did not find statistical differences for height between any of the treatments and the controls. Our results resemble those of Barbosa et al. (2018) who also did not find differences for height in yerba mate seedlings fertilized with phosphorus. Like them, we propose that this could be due to the intense lateral branching of the seedlings, which affected the growth of the main stem.

Although none in yerba mate, several *in vitro* and *in vivo* studies have evaluated the potential of *Bacillus* spp. in promoting the growth of different crops. Galelli et al. (2015) found an increase of 64 % in the weight of the shoots and 68 % for the roots in lettuce (*Lactuca sativa*) inoculated with a PGPR *Bacillus subtillis* (planktonic form). Furthermore, the authors tested the effect of biofilms as a strategy to increase the contact between the bacteria and the plant and found an increase in growth for the shoots of 39 % and 59 % for the roots. Keeping in mind the importance of plant microbe interaction, we have focused on studying endophytic bacteria which have inherent ability to colonize plant tissue to establish an intimate contact with the plant tissues.

Pandey et al. (2018) inoculated amaranth (*Amaranthus hypochondriacus*) seeds with 6 bacterial isolates, two of which were identified as *Bacillus pumilus* and one as *Bacillus subtilis*. These authors found a maximum average enhancement of 85 % in root dry weight and 58.12 % in seedling length compared to a negative control. Like us, they studied endospore-forming bacilli but instead of endophytes they used bacteria isolated from nonrhizospheric soil samples, and contrary to our study they did find

differences for seedling height compared to the negative control.

Sivakumar et al. (2012) inoculated rice with *Bacillus cereus* TS1 and observed height values 108-23 % and 34.36 % higher than the negative control for the dry weight of roots and shoot, respectively, much lower than what we observed for yerba mate seedlings. However, unlike us, they did observe a difference for the height, representing an increase of 15.46 %.

In this way, we developed the present study because of the economic importance that yerba mate holds in our region, which makes it a widely studied and characterized plant with the aim to improve its yield and phytomedicinal properties (Gaiad et al. 2006). Rolim Borges et al. (2011) studied microorganisms found in a yerba mate monoculture focusing on fungi. Contrary to our work, they used soil samples, which contain microorganisms that are not necessarily endophyte and focused on microorganisms with phytopathogenicity potential. However, we emphasize to date there are no studies which report endophytic spore-forming bacteria with PGP properties in yerba mate, except related research by our team (Bergottini et al. 2015a).

Even though PGP strains are often selected based on *in vitro* tests, they do not necessarily correspond to the mechanisms that influence growth promotion *in vivo*. However, the selected strains were able to promote the growth of yerba mate seedlings under controlled laboratory conditions.

Importantly, the bacteria isolated in this study are endophytes and therefore they are in intimate relation with the tissues of yerba mate. In addition, their ability to form spores would make it feasible for them to remain in the crops after inoculation even in dry conditions and high temperatures, decreasing the number of applications needed to obtain improvements in growth.

On the other hand, the formulation of a specific biofertilizer for yerba mate contributes to the development of sustainable agriculture in our region and promises to be a strategy that reduces the environmental impact of agrochemicals.

In fact, 19RS3 and T5S-T4 are excellent candidates for the development of biofertilizers that can replace or efficiently complement the products that are available on the market. We must continue with studies that validate their potential to promote growth in yerba mate in nursery and in the field.

CONCLUSIONS

Thirty-six endophytic spore-forming bacteria were isolated from yerba mate seedlings. Out of 47 studied isolates (36 recovered in this work and 11 collection isolates) 38 were capable of fixing nitrogen, making this the main property detected in our isolates. The second most common property was siderophores production, with 37 isolates positive for this test. Sixteen isolates were able to solubilize phosphate in the *in vitro* test. The 33 isolates tested for indolic compound synthesis showed values of concentration between 0.34 and 4.82 μgmL^{-1} at 72 h.

Three endophytic spore formers with all the tested PGP traits were molecularly identified to be *Bacillus circulans* (12RS3) and *Bacillus altitudinis* (T5S-T4 and 19RS3).

Yerba mate seedlings under laboratory conditions showed higher root dry weight when inoculated with isolate 19RS3 (*Bacillus altitudinis*) and higher aerial part dry weight with 19RS3 and T5S-T4 (*Bacillus altitudinis*). None of the inoculation treatments showed differences in the seedling height when compared to the non-inoculated control.

Bacillus altitudinis (19RS3 and T5S-T4) was the most promising isolate for its use as a bio-inoculant in yerba mate seedlings.

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