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ORIGINAL PAPER

Diversity of endophytic fungal and bacterial communities in *Ilex paraguariensis* grown under field conditions

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Abstract The composition and diversity of the endophytic community associated with yerba mate (*Ilex paraguariensis*) was investigated using culture-depending methods. Fungi were identified based on their micromorphological characteristics and internal transcribed spacer rDNA sequence analysis; for bacteria 16S rDNA sequence analysis was used. Fungal and bacterial diversity did not show significant differences between organ age. The highest fungal diversity was registered during fall season and the lowest in winter. Bacterial diversity was higher in stems and increased from summer to winter, in contrast with leaves, which decreased. The most frequently isolated fungus was *Fusarium*, followed by *Colletotrichum*; they were both present in all the sampling seasons and organ types assayed. Actinobacteria *r*epresented 57.5 % of all bacterial isolates. The most dominant bacterial taxa were *Curtobacterium* and *Microbacterium*. Other bacteria frequently found were *Methylobacterium*, *Sphingomonas*, *Herbiconiux* and *Bacillus*. Nitrogen fixation and phosphate solubilization activity, ACC deaminase production and antagonism against plant fungal pathogens were assayed in endophytic bacterial strains. In the case of fungi, strains of *Trichoderma*, *Penicillium* and *Aspergillus* were assayed for antagonism against pathogenic *Fusarium* sp. All microbial isolates assayed showed at least one growth promoting activity. Strains of *Bacillus*, *Pantoea*, *Curtobacterium*, *Methylobacterium*, *Brevundimonas* and *Paenibacillus* had at least two growth-promoting activities, and *Bacillus*, *Paenibacillus* and the three endophytic fungi showed high antagonistic activity against *Fusarium* sp. In this work we have made a wide study of the culturable endophytic community within yerba mate plants and found that several microbial isolates could be considered as potential inoculants useful for improving yerba mate production.

Keywords Endophytes · Yerba mate · Fungal diversity · Bacterial diversity · PGPB

Introduction

Yerba mate (*Ilex paraguariensis*) is a dioecious evergreen subtropical tree; it can reach a height of 30 m although in production fields it remains as a shrub due to regular pruning. After industrial process, yerba mate leaves are used to prepare a tea or infusion named "mate" that is typically consumed in southern South America, including Argentina, Paraguay, Uruguay and Brazil. The particular flavor and stimulating properties of the yerba mate, given by the caffeine and theobromine content (Filip et al. 2001; Schinella et al. 2005), make it preferred to coffee or tea in many cases. This is the only species of genus *Ilex* that is grown for industrial purpose. Argentina is the main yerba mate world producer with 62 % of total production, followed by Brazil with 34 % and Paraguay with 4 %. In Argentina, the crop is grown in the northeastern provinces of Misiones and Corrientes where subtropical climate prevails (Lysiak 2012a, b).

Living plants provide a habitat for microorganisms which can be found both as epiphytes on the plant surface

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and as endophytes within plant tissues. Endophytes can be defined as those organisms that live in association with plants for most if not all their life cycles, and can be distinguished from other plant associated microorganisms based on the recovery from surface-sterilized plant material (Bacon et al. 2002). Endophytic fungi and bacteria have been found in almost all studied herbaceous or woody plant species (Saikkonen et al. 1998; Bacon and Hinton 2006; Porras-Alfaro and Bayman 2011). The scarce cases of plant hosts apparently lacking endophytic communities might respond to culture or technique failure (Rosenblueth and Martínez-Romero 2006). Endophytes have been found in most plant organs; they can enter the plant through the roots and remain there or become systemic and colonize stem, leaves and even flowers and fruits. However, roots are not the only way of entrance, many phyllosphere endophytes enter the plant trough stomata or wounds (McCully 2001; Schulz and Boyle 2006; Danhorn and Fuqua 2007).

Many endophytes have been shown to have plant growth promoting activities, such as nitrogen fixation (Sevilla et al. 2001; Hurek et al. 2002; Iniguez et al. 2004), production of plant growth regulators (Hamayun et al. 2009), plant protection by production of antibiotic (Bangera and Thomashow 1996), antifungal substances (Hanada et al. 2010) and siderophores (O'Sullivan and O'Gara 1992; Bartholdy et al. 2001), and by systemic resistance induction (Madhaiyan et al. 2004). Although strict endophytes establish non-pathogenic long term association with the plant host, some fungi and bacteria might have a latent or inactive phase, and when the interaction becomes imbalanced either disease results or the pathogen is killed (Schulz and Boyle 2006). Studies have demonstrated that the endophytic community lives in close relation with the plant host influencing physiological processes and interaction with other organisms. Therefore, changes in endophyte composition and diversity might affect plant growth and development. In the case of yerba mate, the identification of bacterial and fungal endophytes will be of great use for developing crop management technologies and improving the species industrial capacity and quality.

There are a few reports of yerba mate associated endophytes. Two endophytic bacteria have been identified, one as in vitro tissue culture contaminant and the other isolated from roots (Collavino et al. 2010; Luna et al. 2013). Endophytic phyllosphere fungi have been described in disinfected leaves from Brazil (Pimentel et al. 2006). There are no reports, however, on the distribution of the endophytic community within the plant nor the variation along the year. The aim of this work was to identify and characterize the culturable endophytic community recovered from surface-disinfected leaves and stems of yerba mate grown under field condition.

Materials and methods

Biological material

The diversity of endophytic fungi and bacteria was estimated in young and mature leaves and stems of three yerba mate (*Ilex paraguariensis*) genotypes (SI-49, LM y G-18), from a commercial breeding program (Las Marías Co.) characterized mainly by their ecophysiological features and yield. Mature branches had secondary growth (woody stems), coriaceous and dark leaves while young ones had herbaceous stems and light green leaves. Samples were collected in triplicate in summer 2010, fall 2010, summer 2011, fall 2011 and winter 2011 from an orchard stand of 8000 plants/ha (close canopy structure) localized in Las Marías Co., Gdor. Virasoro, Argentina (28° 02′ S, 55° 54′ W). The site is characterized by a mean annual rainfall of 1800 mm, distributed mainly during spring and autumn; mean year temperature of 20 °C while frosts are scarce. The soil is described as Ultisol. Three young and mature yerba mate branches (30–60 cm long) were harvested from three random selected plants of each genotype (since no differences were detected among genotypes, samples from the nine plants were analyzed without discriminating genotype) and carried to the laboratory in polyethylene bags at 12–15 °C and immediately processed.

Endophytic microorganisms isolation

Leaf and stem samples were washed with tap water and disinfected in 20 % bleach solution (1.1 % sodium hypochlorite) for 20 min after 2 min in 70 % ethanol, and rinsed three times with sterile distilled water (Sansberro et al. 2000). Two leaf (2 cm^2) and stem (1 cm) sections were cut from each branch for bacteria and fungi isolation. Efficiency of surface sterilization was assayed by inoculating 100 µl of the last rinse water on bacterial and fungal culture medium, and negative samples were selected for further microorganisms isolation.

Fungi were isolated on potato glucose agar medium (PGA, Britania, Argentina) supplemented with 100 mg l^{-1} streptomycin. In the case of bacteria, plant sections were macerated in sterile microtubes and 70 µl of a 1:10 dilution were plated on Petri dishes with tryptone soybean agar medium (TSA) supplemented with 100 mg l^{-1} cycloheximide. Samples were incubated at 27 °C, until fungal and bacterial growth was noticeable (7–30 and 5–10 days, respectively). When fungal and bacterial growth appeared, the colonies were counted and grouped by their cultural characteristics, and representative isolates were collected, purified and preserved for future analysis. The endophytic

microorganisms obtained in this study have been stored at −70 °C at the Instituto de Botánica del Nordeste (IBONE).

Identification of endophytic microorganisms

Fungi

Isolated fungi were identified based on their micromorphological characteristics on PGA medium, mainly according to Domsch et al. (1980) and Ellis (1971, 1976). Isolates that did not sporulate on PGA medium were cultured on different semi-selective media in order to induce typical growth and fungal sporulation. Fusarium-like isolates were transferred to SNA medium (Spezieller Nährstoffarmer Agar) with filter paper (Leslie and Summerell 2006). Other isolates were transferred to cornmeal agar with sterile soybean stems (Dhingra and Sinclair 1985). Incubation conditions were 12 h light with near ultra violet or black lamps (360 nm highest emission). One representative strain for each morphotype was selected for molecular identification using internal transcribe spacer (ITS) ITS1-5,8S-ITS2 rRNA region sequence analysis.

DNA was directly extracted from fungal mycelium grown in liquid potato glucose medium in a 1.5 tube for 72 h at 25 °C according to Cenis (1992). After centrifugation, the mycelial mat was washed in TE (Tris EDTA) buffer and DNA extracted with 0.5 % sodium dodecyl sulfate (SDS) buffer by crushing with a conical grinder. DNA was precipitated by centrifugation with isopropanol, washed with 70 % ethanol and resuspended in 50 ul of TE. DNA quality and concentration were assessed using a NanoDrop® spectrophotometer. Extractions yielded between 50 and 100 ng μl^{-1} DNA with 1.6–1.8 260/280 ratio. Approximately 20 ng DNA were used for polymerase chain reaction (PCR) amplification of ITS1-5,8S-ITS2 region with primers ITS1 (5′-TCCGATGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The reaction conditions were as follows: 95 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. The amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and observed under UV light. For identification, the PCR fragments were purified using a AccuPrep Gel Purification kit (Bioneer), and DNA sequencing was performed by Sanger method using ITS4 primer at the Instituto de Biotecnología (Centro de Investigación en Ciencias Veterinarias y Agronómicas, CICVyA-INTA, Hurlingam, Buenos Aires, Argentina). Partial ITS1-5.8S-ITS2 rDNA gene sequences retrieved in this study were deposited in the GeneBank database under accession numbers KP195143-89 and KP990803.

Bacteria

The 16S rDNA gene was amplified by PCR from genomic DNA isolated from pure bacterial colonies. For DNA extraction, Chelex 100 chelating resin (Bio-Rad) was used according to Alippi and Aguilar (1998). A pellet was obtained after centrifugation of 200-µl suspension of single colonies in sterile distilled water. The pellet was resuspended in 150 µl of an aqueous suspension of 6 % resin Chelex 100 (Bio-Rad), incubated at 58 °C for 10 min, vortex mixed, incubated at 99 °C for 8 min and vortex mixed again. Bacterial debris and resin were precipitated by centrifugation and 2 µl of the supernatant were used for the PCR reactions. The 16S rDNA was amplified with universal primers rD1 (3′-AAGGAGGTGATCCAGCC-5′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg et al. 1991). The reactions were performed under the following conditions: 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by one cycle of 72 °C for 10 min. Amplified PCR products were visualized by 1 % agarose gel electrophoresis. For identification, the PCR products were purified using PureLink™ Quick Gel Extraction & PCR Purification Kit (Invitrogen, USA) and sequenced by Sanger method using rD1 primer at Macrogen Inc., Korea. Partial 16S rDNA gene sequences retrieved in this study were deposited in the GeneBank database under accession numbers KP195190-258.

Phylogenetic analysis

Nucleotide sequences were compared with NCBI GenBank entries and similarities were determined using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Sequence similarity was calculated after Clustal W multiple sequence alignment using MegAlign program (DNASTAR, Lasergene). Phylogenetic analyses were conducted using MEGA version 6 (Tamura et al. 2013). Consensus cladograms were generated based on the maximum likelihood method, with the reliability provided by 1000 replications bootstrap test.

Diversity analysis

Shannon (H′) (Bowman et al. 1971) diversity index was calculated using the Past 3.01 software package. Diversity indexes were compared by non-parametric Kruskal–Wallis H test considering organ type, age and season, using Infostat v. 2014 (Di Rienzo et al. 2014).

In vitro screening for plant growth promoting activities

In vitro growth promoting activities including nitrogen fixation, phosphate solubilization, ACC deaminase gene detection and antagonism against plant fungal pathogens were assayed in 22 bacterial strains representative of genera *Pantoea*, *Micrococcus*, *Bacillus*, *Rhizobium*, *Herbiconiux*, *Sphingomonas*, *Roseomonas*, *Methylobacterium*, *Curtobacterium*, *Brevundimonas*, *Xylophilus*, *Paenibacillus* and *Mycobacterium*. They were also tested for hypersensitive reaction (HR) on tobacco, which allows detection of some pathogenic traits. In the case of fungi, *Fusarium* isolates were assayed for pathogenicity, and strains of *Trichoderma*, *Penicillium* and *Aspergillus* were also assayed for antagonism against pathogenic *Fusarium* sp.

Nitrogen fixation

Bacteria were tested in their free-living nitrogen fixation ability by growing them on semi-solid nitrogen free NFb medium (Döbereiner et al. 1995) along three successive subcultures at 28 °C.

Phosphate solubilization test

Bacteria were grown on National Botanical Research Institute's Phosphate growth medium (NBRIP) with 5 g l^{-1} of tricalcium phosphate (TCP) as the exclusive P source (Nautiyal 1999) and incubated at 28 °C for 2 weeks. Every 2 days halo size and colony diameter were measured, and the solubilization index (SI) was calculated by subtracting the colony diameter from the halo total diameter. Strains displaying an SI equal to or higher than 1.4 in three replicates were considered efficient P solubilizing bacteria. *Pseudomonas* RHP3 strain was used as positive control (Mehta and Nautiyal 2001).

Detection of ACC deaminase gene

Isolates were tested for the presence of *acd*S gene, required for the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, by specific PCR amplifications from genomic DNA. The PCR-detection of *acd*S was performed using the primers F1936f and F1938r (792 bp) as recommended by Blaha et al. (2006). *Pseudomonas putida* ATCC 17399/pRK-ACC (Grichko and Glick 2001) was used as positive control for *acd*S amplification.

Antagonism assay against Fusarium sp

Bacterial and fungal isolates were tested for antagonism against *Fusarium* sp. by the dual culture technique (Fokkema 1978). The pathogenic *Fusarium* strain had been previously isolated from yerba mate symptomatic stems. A mycelial plug from *Fusarium* sp actively growing on potato glucose agar (PGA- Britania) was taken with a 0.5 cm diameter cork borer and placed off center (1.5 cm from the border) on PGA medium in a 9 cm Petri dish. The potential antagonists were inoculated 5 cm from the *Fusarium* plug equidistant from the opposite border. Bacteria were grown in TSB (Tryptone Soybean Broth) from which 10 µl of a 10^6 CFU ml^{-1} suspension was inoculated; fungal isolates were inoculated in the same way as the pathogenic *Fusarium*. Control plates contained only a 0.5 cm diameter *Fusarium* sp. plug in the same position as in the dual culture plates. Dual cultures were incubated at 28 °C during 14 days. *Fusarium* sp. colony growth was registered 7 and 14 days after inoculation and Growth Inhibition (GI) was calculated according to Fokkema (1978): GI = kr − r1/kr 100 (kr = mean of *Fusarium* growth on control plates from the inoculation point towards the plate center in mm; r1 = *Fusarium* growth on dual-culture plate from the inoculation point towards the plate center in mm).

Hypersensitive response (HR) on tobacco

Bacterial isolates were inoculated into tobacco (*Nicotiana tabacum* L.) leaves to evaluate their ability to induce HR (Klement 1963). The isolates were incubated individually at 28 °C for 24 h in TSA medium. Bacteria suspension was prepared in sterile water, and the concentration of bacterial cells was adjusted using a spectrophotometer to approximately 10^9 CFU ml^{-1} before inoculation into fully expanded tobacco leaves using a hypodermic syringe. The needle was inserted into interveinal regions, and the bacterial suspension was injected into the mesophyll intercellular spaces. A negative control containing only sterile water was included in each leaf. Tobacco plants were maintained in a climatic room with a temperature of 26 °C ± 1 with 15 h photoperiod provided by high-pressure sodium lamps (Vialox®, 400 W, OSRAM GmbH, Germany). A positive HR reaction was recorded when the inoculated leaf tissue collapsed or light brown necrosis occurred within 48 h after inoculation. *Xanthomonas axonopodis* pv. *manihotis* was used as a positive control. Each inoculation was repeated at least three times.

Pathogenicity test of *Fusarium* isolates

Three *Fusarium* isolates related to *F. oxysporum*, *F. lateriticum* and *F. fujikuroi* species were tested for pathogenicity on *Ilex paraguariensis* under greenhouse conditions using 5–6 leaf seedlings grown in pine wood

chips substrate and in vitro conditions using plantlets obtained from embryo culture.

Three inoculation techniques were assayed for seedlings: (1) Inoculation of 1 ml conidial suspension on wounded stems, (2) wounded root soak (over night) inoculation with conidial suspension, and (3) agar plug inoculation (approximately 10 mm of fungal colony) on wounded stems and roots. In vitro plantlets were inoculated with 50 µl of conidial suspension by stem wounding using a sterile syringe. The experimental design consisted of five plants (greenhouse assays) or ten plants (in vitro assays) per treatment, included the mock-inoculated control, with three replicates each. Inoculum consisted of 10^6 conidia ml^{-1} suspension according to Zum Felde et al. (2006). The selected *Fusarium* isolates were grown on PGA plates until they produced sufficient conidia. Under laminar-flow hood, conidia were removed from plates by pouring sterile distilled water onto the agar surface with a flame-sterilized glass scraper. Conidial suspension was filtered through sterile gauze into a sterile glass flask. Concentration was determined using a Neubauer haemocytometer and adjusted to 10^6 conidia ml^{-1} with sterile water. Control plantlets were inoculated with sterile water or agar. In vitro plants were incubated at 27 ± 2 °C in a controlled environment room. Greenhouse conditions consisted in 25–30 °C average temperature and 80 % soil humidity achieved by regular watering. Symptom severity was weekly assayed using a subjective rating scale adapted from La Mondia and Taylor (1987) in which 0 = healthy plant, 1 = stunted or off-color plant, 2 = plants with one symptomatic leaf, 3 = plants with more than one symptomatic leaf, 4 = dead plants. *Fusarium* spp. were re-isolated on PGA medium from inoculated plants.

Results

Fungal and bacterial diversity

A total of 216 leaf and stem samples were collected during six surveys performed in summer and fall 2010, and summer, fall and winter 2011. From which 156 (66 from stems, 94 from leaves) and 193 (126 from stems, 66 from leaves) different fungal and bacterial isolates were obtained, respectively. Fungal and bacterial diversity did not show significant differences between organ age. The highest fungal diversity was registered during fall season and the lowest in winter in both leaf and stem samples, with significant difference between leaves and stems in the fall samples (Fig. 1a). Bacterial diversity was higher in stems and increased from summer to winter (summer < fall < winter), in contrast with leaves, which decreased (summer > fall > winter), showing significant difference in the winter samples (Fig. 1b).

Endophytic fungus composition

Based on morphological an sequence analysis, the endophytic filamentous fungi isolated from yerba mate belonged to the Ascomycetes group, distributed in nine Orders: Trichosphaeriales, Hypocreales, Diaporthales, Eurotiales, Capnodiales, Glomerellales, Pleosporales, Botryosphaeriales and Xylariales.

Exploratory BLAST analysis of ITS1-5.8S-ITS2 rRNA partial sequences (260 bp) retrieved similarity to 13 genera including *Acrocalymma*, *Aspergillus*, *Cercospora*, *Clonostachys* (*Bionectria*), *Colletotrichum*, *Curvularia*, *Fusarium* (*Nectria*), *Guignardia* (*Phyllosticta*), *Microsphaeropsis*, *Neofusicoccum*, *Paecilomyces*, *Penicillium* and *Phomopsis* (*Diaporthe*).

Order Hypocreales represents 57.7 % of the fungal isolates. Most of them (85.5 %) corresponded to *Fusarium* species, which presented typical chlamydospores, microconidia and fusiform- to sickle-shaped macroconidia. According to the phylogenetic tree based on rDNA sequences (Fig. 2), *Fusarium* isolates were grouped into five different clusters, six closely related isolates clustered with *F. fujikuroi*, one isolate was related to *F. proliferatum*, one to *F. oxysporum*, three to *F. solani* and three clustered with *F. lateritium* and *F. xylarioide*. *Fusarium* was the most frequently isolated fungal genus. It was present in all sampling seasons and organ type. Two isolates obtained in fall and winter 2011 from young stems were identified as *Trichoderma* based on morphological characteristics, but could not be represented in the phylogenetic tree due to sequences low quality.

Order Glomerellales was represented by *Colletotrichum* with 14 isolates that produced long conidiophores with hyaline single-celled ovoid conidia when grown on PGA medium. Phylogenetic analysis showed that one isolate is closely related to *C. incarnatum* while the rest formed a cluster with *C. boninense* and *C. gloeosporioides* (Fig. 2). They were found on either young or mature leaves and stems in all sampling dates.

Order Diaporthales. Isolates in this order also corresponded to one genus, *Phomopsis/Diaporthe*. They represented 6.4 % of all fungal isolates, and were obtained from leaf and stem samples in summer and fall 2010. In all cases, asexual morphs could be observed in the colonies growing on PGA. Structures where mainly dark pycnidia with hyaline single-celled conidia; in some cases fusiform (alpha) and curved (beta) conidia could be observed. The isolates were separated in four groups according to the phylogenetic tree, which clustered with sequences from

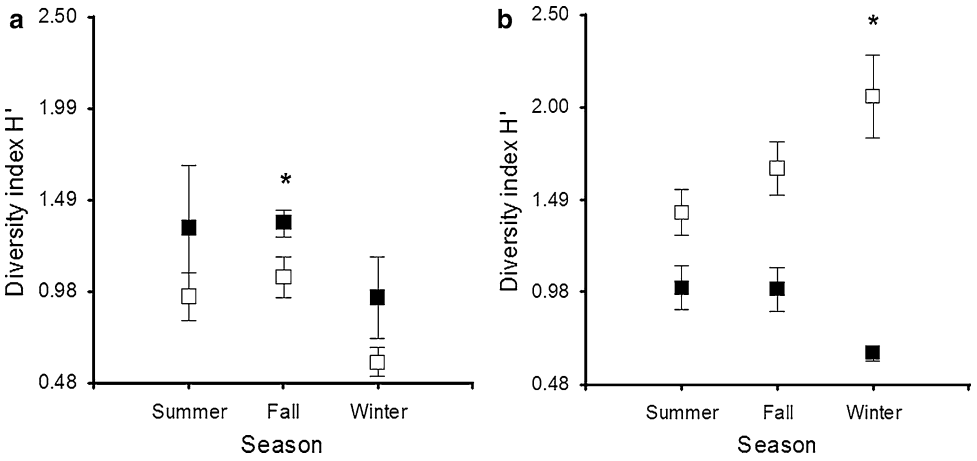


Fig. 1 Diversity of endophytic fungi (**a**) and bacteria (**b**) isolated from yerba mate leaves (*black squares*) and stems (*white squares*). Shannon index (H′) was compared considering organ type and season using non-parametric Kruskal–Wallis H test. * indicates significant difference ($p = 0.05$) between leaf and stem samples in one season. *Bars* indicate standard error

D. phaseolorum and *P. euphorbiae*, *D. paranensis*, *P. longanae*, and *D. heveae* (Fig. 2).

Order Eurotiales. The second most represented order was Eurotiales with 20 isolates (12.8 %), 16 of which were identified as genus *Penicillum*, 3 as *Aspergillus*, and only one isolate corresponded to *Paecilomyces*. *Penicillum* isolates, found in all types of samples from summer 2010/2011 and fall 2011, were related to species *P. citrinum*, *P. citreonigrum* or *P. oxalicum*. *Aspergillus*, on the other hand, was isolated only from young or mature stems in fall 2011.

Order Capnodiales was represented by seven close related isolates (4.5 %), which clustered in the phylogenetic tree with *Cercospora capsicigena*, *C. kikuchii* and *C. canescens* species. They were obtained from fall and summer 2011, young and mature leaf and young stem samples. The same amount of isolates corresponded to order Botriosphaerales, among them were identified genera *Neofusicoccum* (*N. kwambonambiense* and *N. brasiliense*) *and Phyllosticta/Guignardia* (*P. elongata* and *G. mangiferae*).

Less represented orders were Pleosporales with two isolates related to *Curvularia* (*C. lunata*, *C. australiensis* and *C. crepinii*), which were found only in summer 2010 on young leaf samples, and *Microsphaeropsis arundinis*, obtained from fall 2011 samples, and order Xylariales with only one *Eutypa* isolate.

Endophytic bacterial composition

A total of 193 bacterial isolates were identified at least at the genus level by 16S rDNA sequence analysis. Sequences of the bacteria isolated from yerba mate were preliminary compared with the Genbank database by BlastN analysis. After that, sequences where aligned and phylogenetic trees generated by maximum likelihood in which representative bacteria sequences were included.

Actinobacteria represented 57.5 % of all bacterial isolates. The remaining isolates were related to Firmicutes (26.9 %), Alphaproteobacteria (11.9 %) and Gammaproteobacteria (3.6 %). The most numerous genera within Actinobacteria were *Curtobacterium* and *Microbacterium*. *Curtobacterium* was the most numerous genus with 46 isolates obtained from all types of organ during all sampled seasons. In the phylogenetic tree (Fig. 3), the isolates were related with *Curtobacterium flaccumfaciens*, *C. pusillum* and *C. albidum*. The other group of numerous isolates (42) obtained from all sample types was associated with *Microbacterium*. The isolates were grouped into five clusters related to *Microbacterium paludicola*, *M. trichotecenolyticum*, *M. pumilum* and *M. arborescens*, *M. maritypicum* and *M. oxydans*, and *M. testaceum*.

Other isolates (27) associated with class Actinobacteria corresponded to genera *Herbiconiux*, *Kineococcus*, *Leucobacter*, *Micrococcus*, *Mycobacterium* and *Aeromicrobium* (Fig. 3). The strains related to *Herbiconiux* sp. were isolated in all sampling dates from all organ types except for young leaves. Only one strain was obtained for each of genus *Kineococcus*, *Leucobacter*, *Mycobacterium* and *Aeromicrobium*. One strain related to *Kineococcus rhizosphaerae* was isolated from mature stems, and one related to *Leucobacter tardus* was found on mature leaves; both of them were present only during the fall seasons. Another strain, related to *Aeromicrobium alkaliterrae*, was found only in older stems during the winter season. *Mycobacterium*, on the other hand, was obtained from all types of organs in summer 2010. Three strains were related to different species of *Micrococcus*, *M, indicus*, *M. luteus*, and *M. yunnanensis*. All of them were found only during the fall season.

Firmicutes was represented by nine strains related with *Bacillus*, *Staphylococcus*, *Lysinibacillus* and *Paenibacillus* species (Fig. 3). Seven strains were related to *Bacillus* species. Among them, one strain each clustered with

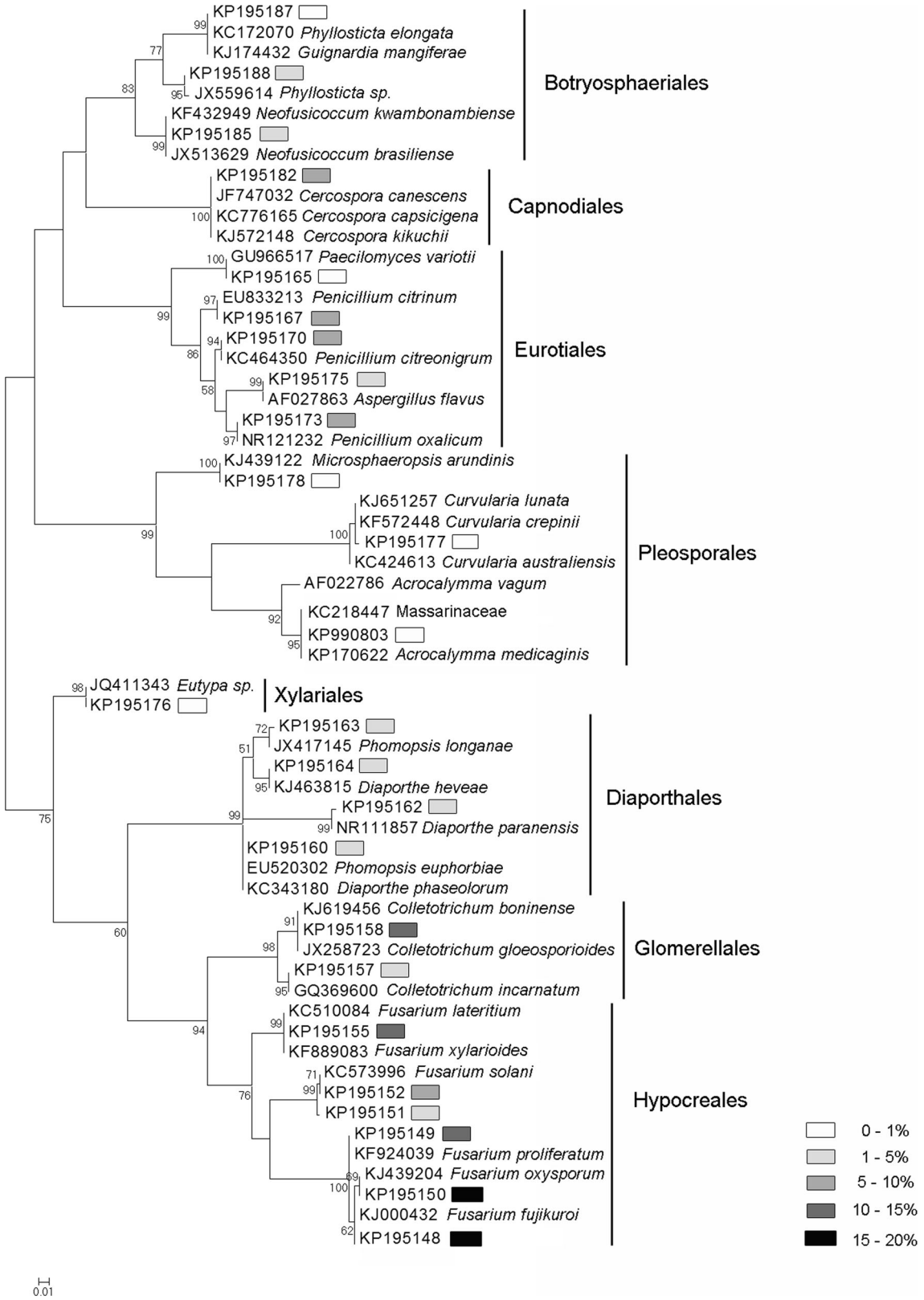
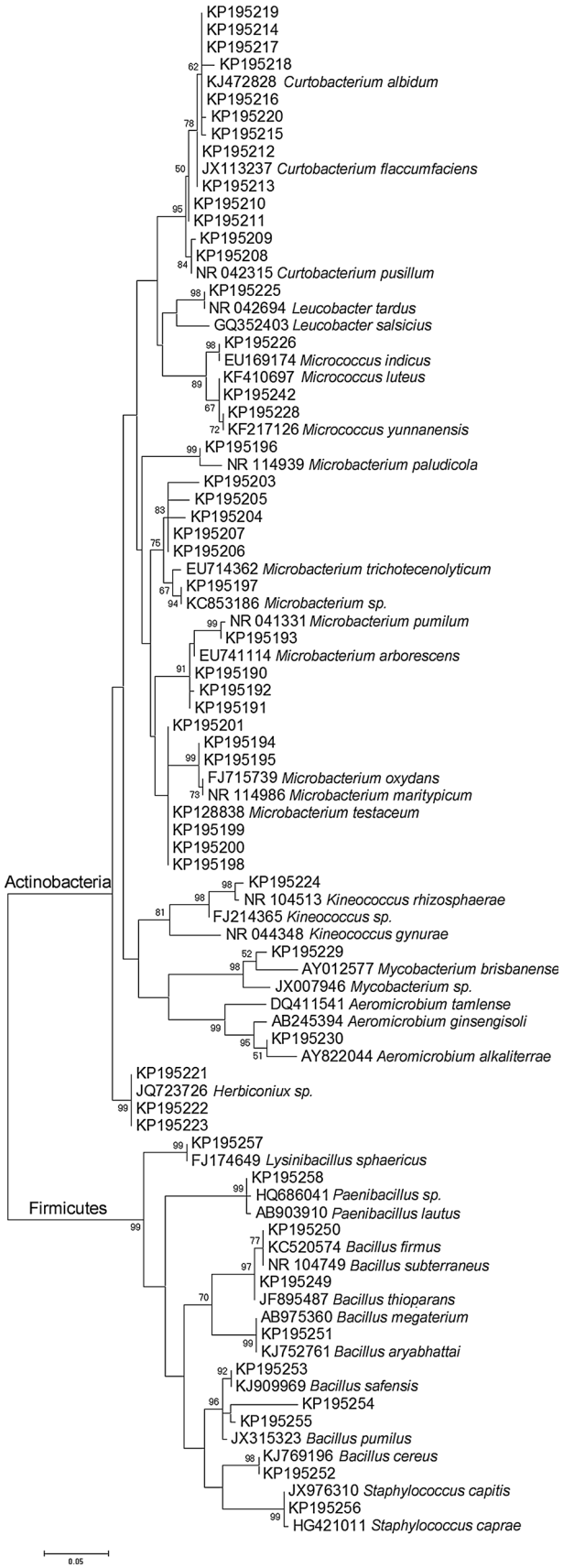


Fig. 2 Phylogenetic tree constructed by maximum likelihood phylogenetic inference based on s ITS1-5,8S-ITS2 region partial sequences including endophytic fungi isolated from yerba mate (*I. paraguariensis*) and closest related genus or species representative. Bootstrap values are shown on *branches* (1000 replications)



◀**Fig. 3** Actinobacteria and Firmicutes phylogenetic tree constructed by maximum likelihood phylogenetic inference based on 16S rRNA partial sequences including endophytic bacteria isolated from yerba mate (*I. paraguariensis*) and closest related representative species or genus. Bootstrap values are shown on *branches* (1000 replications)

B. thioparans, *B. subterraneus* and *B. firmus*, *B. aryabhattai* and *B. megaterium*, *B. cereus*, and three strains clustered with *B. pumilus* and *B. safensis*. Genera *Staphylococcus*, *Lysinibacillus* and *Paenibacillus* were represented by a single strain each, associated with *Staphylococcus caprae* and *S. capitis*, *Lysinibacillus sphaericus*, and *Paenibacillus lautus*.

Alphaproteobacteria was represented by 16 strains, associated with *Methylobacterium*, *Sphingomonas*, *Rhizobium*, *Phenylobacterium*, *Brevundimonas* and *Roseomonas* species (Fig. 4). *Methylobacterium* was represented by five strains obtained from all organ types and sampling seasons. They were associated with *M. brachiatum*, *M. aquaticum*, *M. jeotgali*, and *M. phyllosphaerae*. *Sphingomonas* strains were also present in all seasons and organ type. Three strains could be related to *Sphingomonas* sp. The order Rhizobiales was represented by two strains related to *Rosemonas* sp., found only during the fall season in old leaves and young stems, and four *Rhizobium* sp. strains that were obtained in all seasons only from young organs. Caulobacterales was represented by *Phenylobacterium haematophilum*, *Brevundimonas diminuta*, *B. nasdae* and *B. subvibrioides*. Only two strains were associated with class Gammaproteobacteria (*Xylophilus* sp. and *Pantoea* sp.).

In vitro screening for plant growth promoting (PGP) activities

Nitrogen fixation

Isolates of genus *Micrococcus*, *Bacillus*, *Methylobacterium*, *Curtobacterium*, *Paenibacillus*, *Brevundimonas*, *Roseomonas* and *Mycobacterium* were able to grow on nitrogen free medium, indicating free-living nitrogen fixation capability (Table 1).

Phosphate solubilization test

Seventeen bacterial isolates were able to grow on NBRIP medium with TCP as only P source, indicating P solubilization capability (Table 1). Seven of them were efficient solubilizers (SI higher than 1.4). Among them, *Pantoea* and *Bacillus* isolates were considered early solubilizers since they had significant solubilizing activity during the first hours of culture, with maximal activity within the first

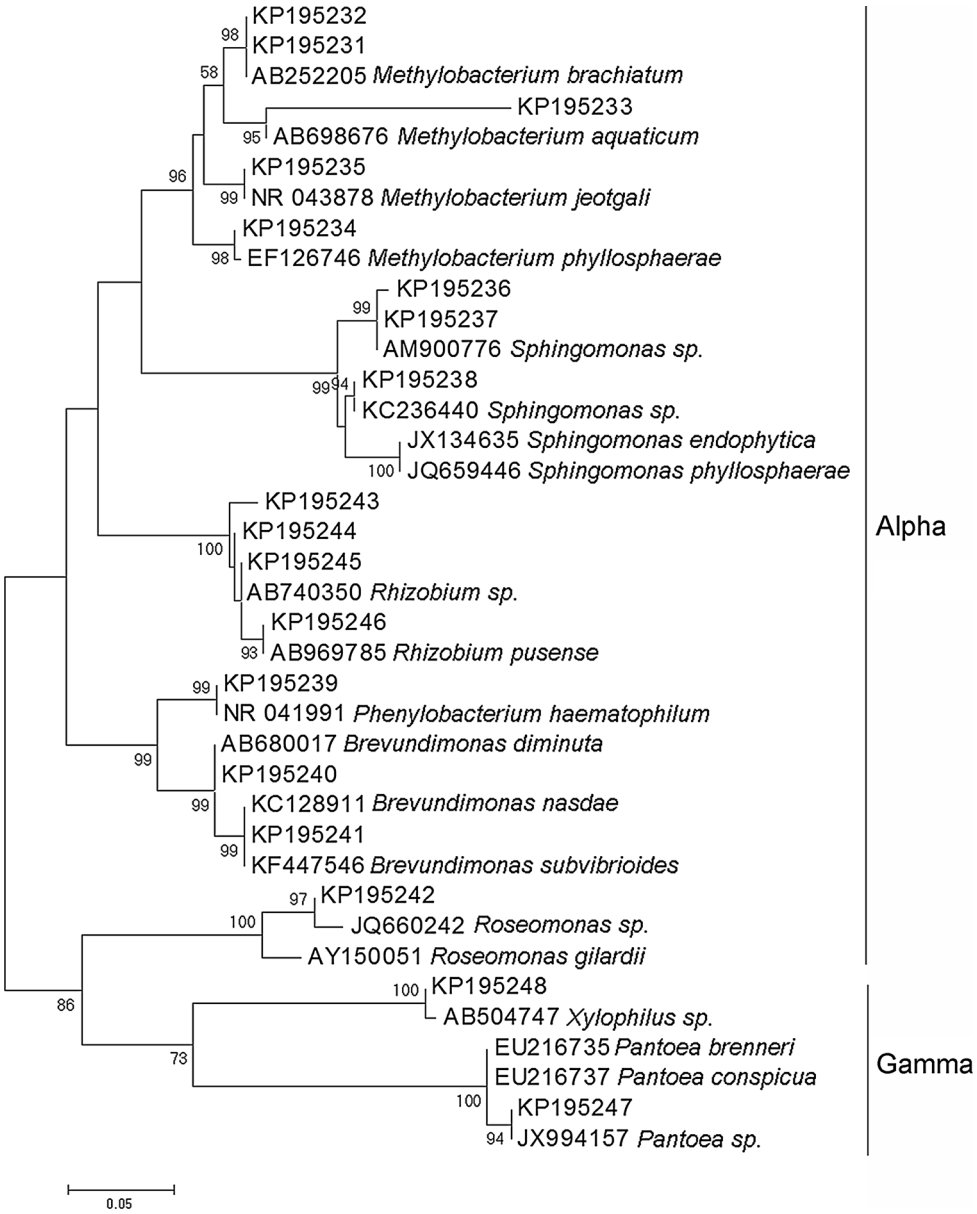


Fig. 4 Proteobacteria phylogenetic tree constructed by maximum likelihood phylogenetic inference based on 16S rRNA partial sequences including endophytic bacteria isolated from yerba mate (*I. paraguariensis*) and closest related representative species or genus. Bootstrap values are shown on *branches* (1000 replications)

72 h of growth. *Curtobacterium*, *Methylobacterium* and *Brevundimonas* isolates, on the other hand, were late solubilizers, showing low activity during the first 72 h and reaching maximal activity between the fourth and fifth days.

Detection of ACC deaminase gene

All the endophytic bacteria assayed resulted negative for the PCR detection of *acd*S gene while the reference strain produced the expected 792 bp band (Table 1). Bacterial DNA proved to be amplifiable by PCR-analysis for 16S rRNA gene using universal primers rD1-fD1 (Weisburg et al. 1991).

Antagonism assay against Fusarium sp

All the isolates assayed showed some level of growth inhibition of the *Fusarium* sp colony when co-cultured, except for *Curtobacterium* ga and both *Methylobacterium* isolates which inhibited less than 5 % (Table 1). The most efficient antagonists strains were *Bacillus* 1N (100 % GI, 7 days after inoculation) and *Paenibacillus* 1o (58 % GI), followed by *Rhizobium* Mle, *Bacillus* 18 and 12b, and *Pantoea* ao2 (GI between 25 and 50 %). The other isolates showed GIs between 1 and 25 %. The three fungal isolates of *Trichoderma*, *Aspergillus* and *Penicillium* inhibited up to 65–75 % of *Fusarium* mycelium growth 7 days after

Table 1 Growth promoting activities of selected yerba mate microbial isolates

Isolate name (Accession number)	Organ source[a]	Fungal growth inhibition (%)[b]	Phosphate solubilization activity (SI)[d]	Free-living nitrogen fixation[e]
Bacillus 2K (KP195254)	*S*	23.6	1.25	+
Bacillus 1N (KP195255)	*S*	100	2	+
Bacillus 12b (KP195253)	*L*	49	2	+
Bacillus 3a (KP195249)	*L*	16.4	0	+
Bacillus 18 (KP195252)	*S*	29	0	−
Bacillus Bc3 (KP195251)	*L*	16.3	1	+
Brevundimonas 2t (KP195241)	*S*	9.1	3	+
Curtobacterium ga (KP195219)	*S*	9	2	+
Curtobacterium 2A (KP195220)	*S*	0	2	+
Herbiconiux 8da (KP195221)	*S*, *L*	23.6	1	+
Methylobacterium R1 (KP195232)	*S*	3.6	1	+
Methylobacterium Rc3 (KP195231)	*S*, *L*	3	1.75	+
Micrococcus Bc7 (KP195226)	*S*	16.4	1	−
Micrococcus af3 (KP195228)	*L*	5	1	+
Mycobacterium 7 (KP195229)	*S*, *L*	16.3	1	+
Paenibacillus 1o (KP195258)	*L*	58.2	0	+
Pantoea ao2 (KP195247)	*L*	25.4	2.6	−
Rhizobium 10h (KP195245)	*L*	14.5	1	−
Rhizobium Mle (KP195243)	*S*	43.6	1	−
Roseomonas 19q (KP195242)	*S*	16.4	1	+
Sphingomonas 27 (KP195236)	*L*	20	0	−
Xylophilus 15L (KP195248)	*L*	18.2	0	+
Trichoderma (KU323643)	*S*	66.7[c]	nd[f]	nd
Aspergillus (KP195175)	*S*	70.4[c]	nd	nd
Penicillium (KP195172)	*L*, *S*	74.1[c]	nd	nd

[a] *L* leaf, *S* stem

[b] Growth inhibition (GI) 14 days after culture against *Fusarium* sp. in dual cultures

[c] For fungal isolates, values represent GI 7 days after culture since antagonists grew over the *Fusarium* colony

[d] Maximal P solubilization activity in NBRIP medium supplemented with tricalcium phosphate. SI of reference strain *Pseudomonas* RHP3: 2.2

[e] Growth on semi-solid nitrogen free NFb medium. +: positive, −: negative

[f] *nd* not assayed

inoculation, and continued growing over the *Fusarium* colony.

Pathogenicity test of *Fusarium* isolates

The three *Fusarium* strains were negative for pathogenicity when tested on greenhouse growing yerba mate seedlings. Different results were obtained when in vitro growing plantlets were inoculated. In such conditions the tree isolates were able to produce disease symptoms, including leaf yellowing, leaf and roots dead, and whole plant dead (Online resource 1). All inoculated isolates reached severity levels of at least three according to the scale (plants with more than one symptomatic leaf) after 49 days, with 50–60 % plantlets dead. Internal and external mycelial growth was observed in symptomatic plantlets, and in all cases the fungus was re-isolated and identification coincident with the inoculated strain.

Discussion

In this work we identified fungal and bacterial endophytes that inhabit yerba mate (*Ilex paraguariensis*), and analyzed the distribution within aerial organs and seasonal variation, considering endophytes as the culturable community

recovered from surface-disinfected plant material. In a previous work, Pimentel et al. (2006) had reported the endophytic fungal population in cultivated and native yerba mate leaves in Brazil. In leaf samples from cultivated plants they recognized five fungal genera (*Aspergillus*, *Colletotrichum*, *Fusarium*, *Penicillum* and *Trichoderma*). In the present work we identified the same fungi and nine other not reported previously (*Acrocalymma*, *Cercospora*, *Clonostachys*, *Curvularia*, *Guignardia*, *Microsphaeropsis*, *Neofusicoccum*, *Paecilomyces* and *Phomopsis*). Besides, endophytic isolates of *Aspergillus*, *Penicillium* and *Trichoderma* identified in this work showed antagonistic activity against *Fusarium*, a typical soil pathogen that affects yerba mate nurseries, and represent possible biological controllers for this species.

We also identified 20 taxa of endophytic bacteria isolated from yerba mate leaves and stems. Recently, two endophytic bacteria have been reported in yerba mate, *Enterobacter* sp. (Collavino et al. 2010) and *Stenotrophomonas malthophilia* (Luna et al. 2013). We did not find such bacteria in our surveys, which might respond to different organ source and plant growth conditions since *Enterobacter* sp. has been isolated from roots and *S. malthophilia* from stems of greenhouse growing plants. On the other hand, we have isolated *Pantoea* sp., which has been previously reported associated with yerba mate rhizosphere (Collavino et al. 2010). The differences registered in bacterial composition and ecological niche suggest that, except for *Pantoea* sp., endophyte establishment in yerba mate aerial organs might have happened independently from root colonization. Further investigation would be necessary to find out the source and colonization means of the yerba mate aerial endophytic bacteria.

Seasonal variation of endophytic communities has been reported in several plant species showing the influence of factors such as environmental temperature and humidity as well as host plant growth dynamics (Osono 2008; Jumponen and Jones 2010). Ding et al. (2013) proposed that plant leaf-associated bacterial communities vary in response to inner biochemical environment changes of the host plants as well as weather and overall environment variations. As reported in other woody species (Kim et al. 2013; Scholtysik et al. 2013), our results showed that fungal diversity begins to increase in the summer reaching the highest values during the fall season. Considering that yerba mate is grown in a climate with rainy fall and spring seasons, the increasing humidity might be one the factors that favor endophytic fungal growth. Yerba mate bacterial endophytic community varied depending on the organ source. The highest stem bacterial diversity was registered in winter as well as the lowest diversity of leaf samples. These results might be related to low diversity of bacteria in the phyllosphere due to lack of nutrients, UV radiation and desiccation (Lindow and Brandl 2003). Variation of bacterial composition as affected by plant tissue has been reported in several plant hosts (Ma et al. 2013; Jin et al. 2014).

Several studies have revealed age-dependent variations of endophytic communities. Species richness of *Camellia japonica* endophytic fungi was low at leaf emergence and increased as leaves aged (Osono 2008), and similar results were found in *Pinus* needles (Guo et al. 2008). Based on such results, many authors supported the early hypothesis of predominantly horizontal transmission of endophytes in trees, as old plant tissues would have had more time to accumulate endophytes from the environment, as opposed to outgrowth from a few initial infection sites (Carroll and Carroll 1978; Guo et al. 2008). In yerba mate, no significant differences were found between young and older leaves and stems although the highest number of fungal and bacterial isolates came from mature leaves and stems, respectively. The crop management practices used in yerba mate fields might explain the low variation of the endophytic community. Yerba mate leaves are harvested three times a year, in June, July–August, and September in such way that the oldest branches and leaves are no more than 1 year old.

Most of the endophytic fungal genera that were isolated and identified in yerba mate have been reported previously as endophytes of woody plants in several geographical regions (Fisher et al. 1993; Suryanarayanan and Vijaykrishna 2001; Sette et al. 2006; Hanada et al. 2010). The most frequently isolated fungus was *Fusarium*; it was present in all the sampling seasons and organ types assayed. Three isolates related to *F. oxysporum*, *F. lateriticum* and *F. fujikuroi* species were tested for pathogenicity on yerba mate plants growing in greenhouse and in vitro conditions. We could not see any disease symptoms on the yerba mate plants from which the samples had been taken; moreover, pathogenicity test performed on pot growing plants was negative. However, when sterile in vitro growing plants were inoculated all three isolates resulted pathogenic and produced symptoms. *Fusarium* species are highly common endophytes, reported in almost all studied host plants. It has even been suggested that endophytism is part of the life cycle of many plant-associated *Fusarium* species (Kuldau and Yates 2000). In support of this hypothesis, endophytic *Fusarium* isolates have been found to be plant pathogens (Kuldau and Yates 2000; Rubini et al. 2005), latent pathogens (Schulz and Boyle 2006; Kharwar et al. 2010), or beneficial with biological control activity (Shiono et al. 2007; Deng et al. 2009). *Colletotrichum* was another frequently found fungal genus. Among others, it has been isolated from *Coffea arabica* and *C. robusta* (Sette et al. 2006), *Ficus benghalensis* (Suryanarayanan and Vijaykrishna 2001), *Camellia japonica* (Osono 2008) and

Theobroma cacao (Hanada et al. 2010). Our results agree with *Colletotrichum* being a typical ubiquitous endophyte since we isolated it from young and old leaves and stems during all sampling seasons.

Among the bacteria isolated from yerba mate, Actinobacteria was the most abundant taxon, in particular the genera *Curtobacterium* and *Microbacterium*, which were isolated from all the organs assayed, showing that they are ubiquitous endophytes. Similar results have been reported in several plant hosts (Zinniel et al. 2002; Thomas et al. 2006; Magnani et al. 2010; Gagne-Bourgue et al. 2013). Plant endosphere is a common actinobacteria habitat, in which the bacteria show different degree of functional and genetic diversity. They play an important role for the host plant by producing high amounts of catabolites and growth promoting compounds (Araujo et al. 2002; Lacava et al. 2007; El-Shatoury et al. 2013; Brader et al. 2014). At least two of the endophytic *Curtobacterium* isolated from yerba mate had the ability to efficiently solubilize phosphates and fixing nitrogen, one of them had also the ability of inhibiting *Fusarium* growth.

Other bacteria frequently found throughout the year were the alphaproteobacteria *Methylobacterium*, and *Sphingomonas*. These genera have been isolated as endophytes elsewhere and proved to have growth promoting activities. Species of genus *Methylobacterium* have been reported to interact as endophytes with several plant species such as *Citrus* spp. (Araujo et al. 2002), *Eucalyptus* sp. (Ferreira et al. 2008) *Coffea arabica* and *Capsicum annuum* (Dourado et al. 2012). Among them, *Methylobacterium nodulans* is involved with nitrogen fixation, as well as some *Sphingomonas* species (Sy et al. 2001; Videira et al. 2009). We detected similar activity in *Methylobacterium* isolated from yerba mate but not in *Sphingomonas*. As regards biological control, isolates from *Sphingomonas* were able to inhibit *Fusarium* growth. However, the endophytic bacteria that most efficiently controlled *Fusarium* in dual cultures were the Firmicutes *Bacillus* and *Paenibacillus*, which were also able to fixing nitrogen and, in the case of *Bacillus*, phosphate solubilizing. *Bacillus* 1N, isolated from yerba mate stems, represents a promising growth promoting bacteria, and will be considered for future *in planta* studies. This is not surprising since both genera have been extensively studied for their growth promoting activities (Bacon and Hinton 2006; Zhao et al. 2014; Rybakova et al. 2015).

In this work we analyzed the culturable endophytic community. We are aware that fastidious or unculturable microorganisms might not be detected by culture-dependent approach. The predominant fungi and bacteria detected in yerba mate were similar to those reported in other works in which the same type of technique was used. Culture-independent methods would provide a better understanding of the endophytic population and their interactions. However, the knowledge of the culturable endophytic community composition is of great value for the development of technologies for agricultural management, in particular those related to microorganism-based growth promotion since the isolates are available for activity and inoculation analyses. In this study, the potential of endophytic isolates for plant growth promotion was determined by assessing nitrogen fixation, phosphate solubilization, ACC deaminase gene detection and fungal antagonism. Apart from *Bacillus* isolates, strains of *Pantoea*, *Curtobacterium*, *Methylobacterium*, *Brevundimonas* and *Paenibacillus* are to be considered as potential bacterial inoculants for having growth promoting activities. In particular, *Bacillus*, *Paenibacillus*, *Trichoderma*, *Penicillum and Aspergillius* are strong candidates for biological control against *Fusarium* sp.. This results could be the bases of developing strategies for improving yerba mate production with minimum needs of agrochemicals and therefor less aggressive with the environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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