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Bio-inoculation of yerba mate seedlings (*Ilex paraguariensis* St. Hill.) with native plant growth-promoting rhizobacteria: a sustainable alternative to improve crop yield

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Abstract In this study, the role of native plant growth-promoting rhizobacteria (PGPR) as bio-inoculants was assessed as an alternative to ameliorate *Ilex paraguariensis* St. Hill. growth in nursery comparing poorer (soil) versus richer (compost) substrates. Twelve rhizospheric strains isolated from yerba mate plantations were evaluated in vitro for their potential as PGPRs. Three isolates, identified as *Kosakonia radicincitans* YD4, *Rhizobium pusense* YP3, and *Pseudomonas putida* YP2, were selected on the basis of their N_2 fixation activity, IAA-like compound and siderophore production, and phosphate solubilization. A highly significant positive effect of bio-inoculation with the native isolates was observed in 5-month-old seedlings cultivated in soil. The highest increase was observed in seedlings inoculated with *K. radicincitans* YD4 with an increase of 183 % in the dry shoot weight and a 30 % increase in shoot N content. In contrast, in compost, no increment in the dry weight was observed; however, an increase in content in some macronutrients in shoots was observed. Remarkably, when plant biomass was compared between soil and compost, seedlings inoculated with *K. radicincitans* YD4 in soil produced the highest yields, even though higher yields could be expected in compost due to the richness of this substrate. In conclusion, bio-inoculation of yerba mate seedlings with native PGPR increases the yield of this crop in nursery and could represent a promising sustainable strategy to improve yerba mate growth in low-fertility soils.

Keywords *Ilex paraguariensis* St. Hill. (yerba mate) · PGPR · Bio-inoculants · *Kosakonia radicincitans*

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Introduction

Yerba mate (*Ilex paraguariensis* St. Hill.), a species of the family Aquifoliaceae, is a native tree of the Atlantic Forest in northeastern Argentina, Paraguay, and South Brazil. This tree is a very valuable regional crop because its leaves are processed into a traditional beverage called mate, consumed as an alternative to coffee by millions of South Americans due to its stimulant effects. Moreover, yerba mate popularity is expanding to new markets including the USA, Europe, and Asia because of its high content of antioxidants and benefits for human health (Heck and De Mejia 2007). Argentina is the principal producer with 90 % of yerba mate plantations (198.932 ha) concentrated in Misiones province. About 50 % of these plantations are currently in a process of degradation. The principal reasons for the decline in productivity

are the age of trees and nutrient depletion due to inadequate management practices (e.g., 80-year-old plantations in continuous monoculture). In Misiones, yerba mate is typically cultivated in Ultisols and Oxisols. However, after a few years of crop production, fertility decreases substantially. This rapid decline in fertility is accompanied by a decrease in the content of soil nutrients such as N, P, and organic matter (Fernandez et al. 1997). Due to the current state of degradation, more sustainable practices have been recommended in yerba mate agricultural systems. Although several approaches have been tested in order to improve productivity and soil quality (Eibl et al. 2000; Ilany et al. 2010), the effect of bio-inoculation with native beneficial bacteria has not yet been explored. Numerous bacterial species, principally those associated with plant rhizosphere or root tissues, have been reported to have a positive impact on plant growth and health (Kloepper et al. 1989; Dobbelaere et al. 1999). These bacteria, collectively known as plant growth-promoting rhizobacteria (PGPR), improve plant growth through different mechanisms as biofertilizers (Kloepper et al. 1980; Cattelan et al. 1999; Rodrigues et al. 2008; Tarnawski 2008), phytostimulators (Mehnaz et al. 2001), or bio-control agents (Compant et al. 2005; Pieterse et al 2009). The rhizosphere and roots of several crops are relevant microniches for the isolation of native PGPR strains adapted to endemic soil conditions from which effective bio-inoculants can be formulated. In this context, PGPR bio-inoculation could represent a promising alternative to produce more vigorous yerba mate seedlings. We hypothesized that native PGPR found associated to roots of the plant will have a positive effect on growth, particularly in poor soil and compared to non-native PGPR strains. Therefore, the aim of this study was to isolate, characterize, and select native PGPR strains associated with yerba mate to assess their effect as bio-inoculants for this crop in nursery.

Material and methods

Isolation of rhizobacteria

Fifty grams of active roots (depth up to 20 cm) from five plants in a productive plantation (YP; 26° 54′ 58.03″ S 54° 25′ 40.47″ W) (20 years old), a soil-degraded plantation (YD; 27° 02′ 58.10″ S 54° 30′ 29.12″ W) (35 years old), and yerba mate trees (YN; 20° 55′ 35.88″ S 54° 25′ 57.79″ W) (40 years old) from the rainforest in San Vicente (175 m.a.s.l), Misiones Province, were collected in winter in July 2010. In YP, the Ultisols contained 32 g kg^{-1} C, 3 g kg^{-1} N, and 1.5 mg kg^{-1} P with a pH of 6.3; in YD, the Ultisols contained 24 g kg^{-1} C, 2 g kg^{-1} N, and 2.9 mg kg^{-1} P with an acidic pH of 4.5; and in YN, the Ultisols contained 734 g kg^{-1} C, 4 g kg^{-1} N, and 0.9 mg kg^{-1} P with a pH of 5.8. Roots were transported to the laboratory (at 4 °C). For bacterial isolation, 10 g of rhizospheric soil obtained by shaking vigorously the roots were dissolved in 90 ml of sterile physiological solution (0.85 % NaCl) and incubated under agitation at 30 °C for 30 min. The soil suspension was filtered (sterile filter paper), diluted tenfold serially, and spread onto nitrogen-free LG media agar plates (Döbereiner 1980). After 48 h of incubation at 30 °C, a bacterial count of approximately 3×10^5 CFU/g was obtained for all the soil types. Four colonies with different morphology were picked per sample and purified by streaking out on tryptic soy agar (TSA) (Difco, USA).

Taxonomical identification

DNA of strains was extracted using the InnuPREP Bacteria DNA Kit (Analytik Jena, Germany). DNA was quantified with a Qubit® dsDNA BR Assay Kit with a Qubit®2.0 Fluorometer (Invitrogen Ltd., UK). PCR amplification of the 16S rRNA gene was performed using the primer sets GM3F/GM4R (Muyzer et al. 1995) and Eub9_27/Eub1542 (Liesack et al. 1991). To obtain the nearly complete 16S rRNA gene sequence, PCR products were sequenced additionally with the primers 338f, 518r, and 926f (Muyzer et al. 1995; Ovreas et al. 1997). The search for similarity among sequences of the 16S rRNA gene was performed using BLASTn (Altschul et al. 1997) with the non-redundant database of GenBank. The 16S rRNA gene sequences from the isolates have been deposited in GenBank under accession numbers KP313536–KP313547.

In vitro plant growth-promoting activity tests

Inorganic phosphorus solubilization was evaluated by the formation of a dissolution translucid halo after 72 h on National Botanical Research Institute's phosphate growth medium (NBRIP) agar plates with $Ca_3(PO_4)_2$ as sole phosphate source (Nautiyal 1999). This trait was verified on solid medium after two consecutive transfers of the cultures. Phosphate solubilization was further verified in the strains after bio-inoculation in solid and liquid NBRIP medium supplemented with either $Ca_3(PO_4)_2$, $AlPO_4$ or $FePO_4$ at a concentration of 5 g L^{-1}. Soluble phosphate in the supernatant was determined by the molybdenum blue method (Murphy and Riley 1962). Synthesis of siderophores was evaluated by the formation of an orange halo around colonies growing in blue agar CAS medium (Schwyn and Neilands 1987). Nitrogenase activity was measured with the acetylene reduction assay (ARA) (Stewart et al. 1967). Activity was analyzed in 20-ml vials containing 8 ml of semi-solid LG medium and 5 ml of a bacterial suspension adjusted to an abundance of 10^8 cells ml^{-1}. After 48 h of incubation, the vials were sealed hermetically and 10 % of the atmosphere was substituted by acetylene. The cultures were incubated for 2 h at 30 °C in darkness prior to measurement. Ethylene production was

measured with a SRI Instruments 8610C gas chromatograph equipped with a HayeSep T column (at 70 °C) and a flame ionization detector (FID at 150 °C). Synthesis of arylsulphatases was evaluated by the formation of a blue coloration around colonies on modified Angle agar plates with 40 mg L^{-1} X-sulfate and 710 mg L^{-1} Na_2SO_4 as sulfur sources (Kertesz and Mirleau 2004). Synthesis of indole-3-acetic acid-like compounds (IAA-like) was estimated by the colorimetric technique specific for the detection of indolic compounds (Bric et al. 1991) as described in Tarnawski et al. (2006). The intensity of the coloration was measured at a wavelength of 535 nm using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific). The value reported was normalized to bacterial growth using cellular abundance measured at 600 nm. Production of hydrogen cyanide (HCN) was tested on a synthetic medium containing glutamate, glycine, and methionine as precursors of HCN (Castric and Castric 1983) as described in Tarnawski et al. (2006). Inhibition of the growth of fungi and protists responsible for damping-off attack (often encountered in plantations and in nursery) was evaluated in a dual culture assay on PDA by inoculating 10 μl of fresh bacterial cultures in triplicate at equidistant points around the perimeter of the plate. A 5-mm plug obtained from a 5-day-old culture of *Fusarium culmorum*, *Rhizoctonia solani*, and *Pythium ultimum* (Collection, University of Neuchâtel) was placed at the center of the plate. Plates were incubated at room temperature, and after 7 days, the inhibition of growth for the damping-off agents was analyzed. Unless otherwise stated, all incubations were performed at 30 °C. A summary of the positive and negative controls for the tests is given in Supplementary Table 1.

Plant bio-inoculation assay

Three native isolates and *Azospirillum brasilense* strain 245 were tested as bio-inoculants in yerba mate seedlings cultivated in nursery. The assay was performed in an organic nursery of yerba mate in Santo Pipó, Misiones, Argentina, from May to November 2013. Yerba mate seedlings with the most homogenous phenotype were selected from the seedbed to be transplanted into pots (200 cm^3) with approximately 150 g of soil (Ultisol) or organic compost. The soil presented a pH of 5.17, 39,100 mg kg^{-1} of C, 3700 mg kg^{-1} of N, 13.94 of C/N, 3.85 mg kg^{-1} of P, and 43 mg kg^{-1} of K and the compost a pH of 5.07, 380,000 mg kg^{-1} of C, 18, 300 mg kg^{-1} of N, 21.14 of C/N, 3033 mg kg^{-1} of P, and 200 mg kg^{-1} of K. Five-month-old seedlings, grown under a shade net were irrigated with 5 ml of a fresh bacterial suspension adjusted to an abundance of 10^8 cells ml^{-1}. Control plants were irrigated with water. The organic compost consisted in a mix 1:1 of composted pine wood chips and rice husk. The experiment was designed as a random block with three replicates of ten plants per treatment. After 6 months post-inoculation, plants were harvested to analyze shoot and root dry weight. As yerba mate seedlings were not homogenous in their height, we normalized the shoot dry weight by seedling height. To obtain an indicative effect of the bio-inoculation on the content of macronutrients in shoots, the 30 plants in each treatment were pooled, dried, ground in a mortar and sieved for subsequent nitro-perchloric digestion. In the extracts obtained, N was measured by the semi-micro-Kjeldahl method; P by the vanadate/molybdate method; and Ca, K, and Mg by EDTA titration.

Statistical analyses

The mean of N_2 fixation rates and production of IAA-like compounds, as well as the effect of bio-inoculation in each separate substrate, were compared by a one-way ANOVA, and pairwise differences were tested using Tukey's post hoc test ($P \leq 0.05$). To examine the differences in plant biomass production between soil and compost, a two-way ANOVA was used and pairwise differences were tested using Tukey's post hoc test ($P \leq 0.05$). Data were analyzed using the R statistics software version 3.1.0.

Results

Evaluation of PGPR properties of the isolates in vitro

Twelve bacterial strains isolated from yerba mate rhizosphere were assigned to six genera: *Kosakonia*, *Pseudomonas*, *Acinetobacter*, *Sphingobium*, *Rhizobium*, and *Ensifer*, on the basis of their partial 16S rRNA gene sequence (Table 1). To select putative PGPR for bio-inoculation assays, these isolates were analyzed for different plant growth-promoting activities in vitro (Table 1). Two isolates, *Kosakonia radicincitans* YD4 and *Pseudomonas putida* YP2, were positive for $Ca_3(PO_4)_2$ solubilization after re-culturing. However, in both cases, this trait could not be reproduced after the bio-inoculation assays both for solid or liquid medium. Seven isolates (YD4, YP3, YP1, YD1, YP4, YD2, and YN4) were positive for siderophore production. Even though the strains were isolated in N-free medium, N_2 fixation was measured quantitatively for the most promising strains. *P. putida* YP2 and *Pseudomonas putida* YN3 displayed a significantly lower activity (P value ≤ 0.05), while the others did not differ significantly in their capability to fix N_2. All isolates produced IAA-like compounds. The highest IAA-like producer was *Rhizobium pusense* YP3, followed by *R. pusense* YP4 and *Acinetobacter radioresistens* YD1. In addition, the isolates were tested for production of arylsulphatases, but only *Sphingobium yanokuyae* YD3 was positive. Bio-control activities were also evaluated. None of the isolates showed the ability to produce hydrogen

Table 1 In vitro plant growth-promoting activities of the isolates

Origin of strain	Strain	Phylogenetic assignment (% identity)	Siderophore production	N_2 fixation (μmol/vial.h)	IAA (μg ml^{-1}/OD600)
Soil-degraded plantation	YD4	*Kosakonia radicincitans* (99 %)	+	42.1 a	24.4 bc
Productive plantation	YP3	*Rhizobium pusense* (99 %)	+	50.7 a	59.2 ab
Productive plantation	YP2	*Pseudomonas putida* (99 %)	–	1.5 b	5.0 d
Soil-degraded plantation	YD3	*Sphingobium yanoikuyae* (99 %)	–	nd	15.0 cd
Productive plantation	YP1	*Rhizobium cellulosilyticum* (99 %)	+	nd	10.7 d
Soil-degraded plantation	YD1	*Acinetobacter radioresistens* (99 %)	+	40.9 a	34.4 bc
Productive plantation	YP4	*Rhizobium pusense* (99 %)	+	nd	43.8 b
Soil-degraded plantation	YD2	*Kosakonia radicincitans* (99 %)	+	42.5 a	24.1 bc
Trees in the rainforest	YN4	*Acinetobacter radioresistens* (99 %)	+	41.1 a	12.3 d
Trees in the rainforest	YN2	*Ensifer adhaerens* (100 %)	–	47.9 a	11.5 d
Trees in the rainforest	YN1	*Ensifer adhaerens* (100 %)	–	nd	6.9 d
Trees in the rainforest	YN3	*Pseudomonas putida* (99 %)	–	2.8 b	3.7 d
	A. brasilense 245		+	39.6 a	76.2 a

nd not determined

Different letters indicate statistical differences in PGPR activities among isolates, investigated by ANOVA followed by Tukey's post hoc test

cyanide. Nevertheless, *S. yanokuyae* YD3 was effective in controlling growth of *Pythium ultimum* in the antagonist test. Based on the results of the in vitro PGPR activities, three isolates were selected: *K. radicincitans* YD4, *R. pusense* YP3, and *P. putida* YP2.

Bio-inoculation assay

Native bio-inoculants had a highly significant positive effect in growth of yerba mate seedlings in soil (Fig. 1). All native strains produced a significant increase on the shoot and root dry weight in comparison to un-inoculated controls. In contrast, seedlings treated with the non-native bio-inoculant (*A. brasilense* strain 245) had lower yields than the control. *K. radicincitans* YD4 was the most effective inoculant with an increase of 183 % on the dry shoot weight and 150 % on the dry shoot weight/height ratio. For *P. putida* YP2, the increase in dry shoot mass corresponded to 102 % and for *R. pusense* YP3 to 92 %. The latter two isolates increased by 67 % in the dry shoot weight/height ratio in comparison to non-inoculated seedlings in soil. No significant increase in plant biomass was observed in compost. The performance of the bio-inoculants was also compared between substrates (soil versus compost) and bio-inoculation treatments. In this global comparison, bio-inoculation with *K. radicincitans* YD4 in soil was the treatment that produced the highest yield overall (P value ≤0.05). Bio-inoculation not only showed a positive effect on yerba mate yield but also appeared to influence the content of macronutrients in the shoot (Supplementary Table 2). In soil, bio-inoculation with *K. radicincitans* YD4 enhanced by 30 % N concentration. However, no increase was observed for the concentration of other macronutrients (P, K, Ca, Mg) in comparison to the controls. The bio-inoculants *P. putida* YP2 and *R. pusense* YP3 did not enhance the concentration of any of the measured macroelements (Supplementary Table 2). In compost, all four bio-inoculants produced significant increases in the content of macronutrients (N, P, K, Ca) compared to controls.

Discussion

In this study, twelve PGPR bacterial strains were isolated from yerba mate rhizosphere. Some of them belonged to well-known plant growth-promoting genera, such as *Pseudomonas* (Kloepper et al. 1989), *Kosakonia* (Peng et al. 2009), *Rhizobium* (Bertrand et al. 2007), and *Ensifer* (Fox et al. 2011). Previously, Collavino et al. (2010) isolated phosphate-solubilizing bacterial strains from the roots and rhizosphere of yerba mate, which were assigned to the genera *Enterobacter*, *Pantoea*, *Pseudomonas*, *Acinetobacter*, *Burkholderia*, and *Exiguobacterium.* In our study, we report other genera of rhizobacteria associated with this crop including *Sphingobium*, *Ensifer*, and *Kosakonia* as potential PGPRs. Although we evaluated PGPR traits including those for bio-fertilization, phyto-stimulation, or bio-control, we selected for the bio-inoculation assay three strains with PGPR traits favoring nutrient acquisition and phytohormone production. The three native strains produced a highly significant increase in biomass yield in soil, in comparison to the non-native PGPR strain *A. brasilense* 245. This points out to the importance of using native strains as effective bio-inoculants (Fages and Arsac 1991). Interestingly, when biomass yields are compared between soil and compost, better yields were obtained in soil

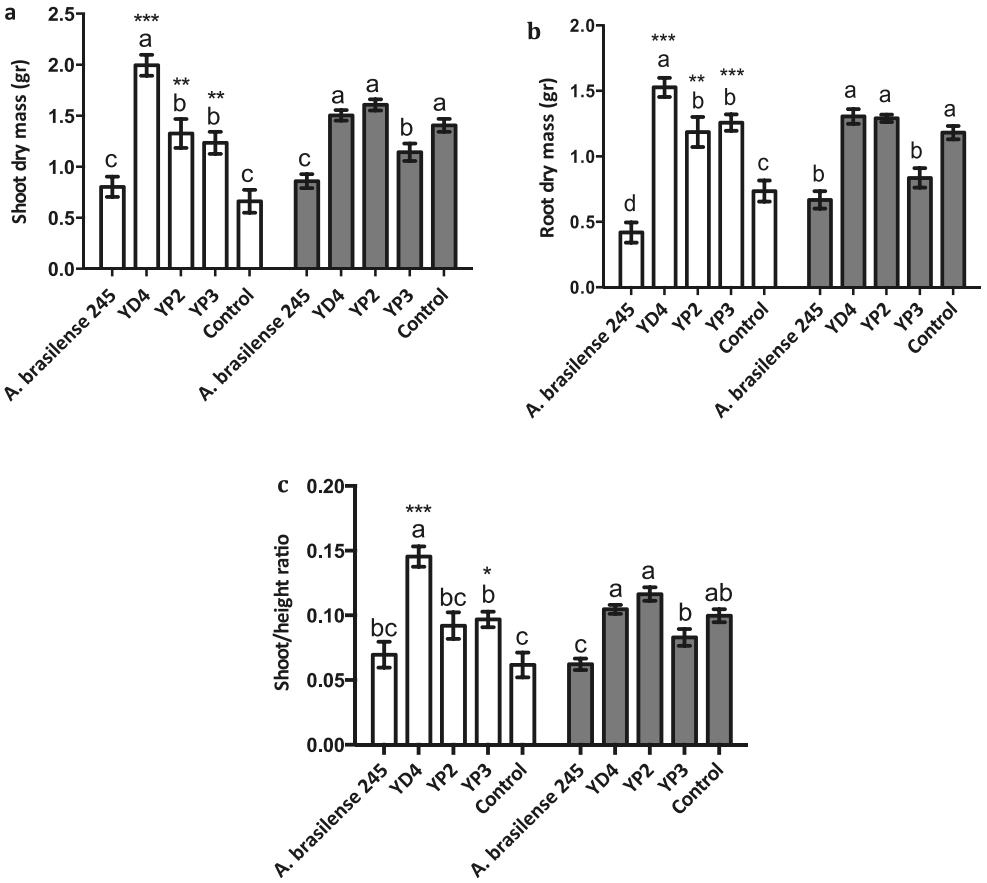

Fig. 1 Effect of bio-inoculation with native rhizobacteria on the growth of yerba mate (*Ilex paraguariensis*) cultivated in soil or compost in nursery. Seedlings were inoculated with selected rhizospheric isolates from yerba mate plantations, *K. radicincitans* YD4, *P. putida* YP2, and *R. pusense* YP3 and with *A. brasilense* 245 strain used as bio-inoculants for several crops. Means of shoot dry mass (**a**), root dry mass (**b**), and shoot/height ratio (**c**) were calculated from 30 plants per treatment after 6 months of cultivation. *White and gray columns* represent means obtained in soil and in compost, respectively. Performances between bio-inoculants and non-inoculated controls were compared within soil and compost separately by one-way ANOVA and Tukey as post hoc-test. Statistically significant differences in bio-inoculant's performances in comparison with non-inoculated controls are indicated by *different letters* ($P \leq 0.05$). Statistical differences between bio-inoculants and the control conditions in soil are shown as $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$

through the bio-inoculation with *K. radicincitans* YD4, even though higher yields were to be expected in compost due to the higher fertility of this substrate. These results are similar to those in which PGPR inoculation alone or associated to mycorrhizae gave more pronounced effects on plant growth promotion in less fertile conditions (Freitas and Germida 1990; Mader et al. 2011). Although PGPR strains are often selected based on in vitro tests, those do not necessarily correspond to the mechanisms influencing growth promotion in vivo (Collavino et al. 2010; Smyth et al. 2011) Nonetheless, it can be hypothesized that the mechanisms that stimulated plant growth are explained by a synergic combination of the PGPR traits identified for each isolate (Bashan et al. 2004). For example, the increase in N content observed in seedlings' shoots in soil inoculated with YD4 could be explained by a better N uptake by the plant due to N_2 fixation by this strain, although a direct transfer of N should be measured to verify this (James 2000). Likewise, the ability to produce IAA-like compounds in stimulating root development and subsequently increases in water and nutrient acquisition could be another mechanism of plant growth promotion (Glick 2012). Finally, production of siderophores could have favored iron facilitation to the plant (Robin et al. 2008) or constituted an advantage for the PGPR strains in colonizing the rhizosphere by depriving the native microflora from iron (Kloepper et al 1980). In the case of the most promising strain, *K. radicincitans* YD4, whole genome analysis revealed the presence of genes potentially involved in PGPR traits such as N_2 fixation (i.e., complete *nif* operon) as well as siderophore and auxin production (Bergottini et al. 2015). A trait that is particularly relevant in agriculture is phosphate solubilization (Bashan et al. 2013a, b). *K. radicincitans* YD4 initially displayed this activity, but it was not detected when checked after bio-inoculation. This was the case even when different metal-phosphate sources were tested as suggested by Bashan et al. (2013a, b). Genes potentially involved in this trait such as those responsible for the production of gluconic acid (pyrroloquinoline-quinone operon) have not been

identified in the genome of *K. radicincitans* YD4. However, we can hypothesize that one of the other mechanisms of phosphate solubilization can be present in this strain (reviewed in Sharma et al 2013a, b). Nonetheless, transfer of phosphate to the plant was neither assessed nor can it be inferred from the nutrient content in shoots, and therefore, this mechanism should be verified in future experiments. *Kosakonia* species, previously classified into the genus *Enterobacter* (Brady et al. 2013), have been reported as PGPR in rice (Peng et al. 2009), groundnut (Madhaiyan et al. 2010), and winter wheat (Witzel et al. 2012). Previous studies showed that the production of phytohormones and N_2 fixation were the main plant growth-promoting mechanisms in winter wheat (Scholz-Seidel and Ruppel 1992), while in *Arabidopsis thaliana*, *K. radicincitans* not only enhanced plant growth but also induced priming of the immune response (Brock et al. 2013). In addition to the mechanisms already cited, a synergistic rhizobacteria-mycorrhizae interaction could have also played a key role. Yerba mate has symbiotic associations with arbuscular mycorrhizae (Andrade et al. 2000), and mycorrhizae-colonized roots were observed microscopically in seedlings in the plant assay (data not shown). The positive effect of synergistic rhizobacteria-mycorrhizae interactions has been demonstrated (Chanway and Holl 1991; Sing and Adholeya 2003; Mader et al. 2011), and this should be tested in the future in yerba mate. In conclusion, this study assessed for the first time the effect of native plant growth-promoting rhizobacteria on the growth of yerba mate seedlings in nursery. Albeit further experiments need to be performed under field conditions, the potential of our isolates as PGPR was demonstrated by higher yields of inoculated yerba mate seedlings in soil. These results are encouraging to continue with the isolation and selection of promising plant growth-promoting bacterial strains for this crop. A test with unspecific bacterial markers was performed to track the inoculants in the rhizosphere, but the results were inconclusive. Therefore, complementing the current study with the analysis of the genome of YD4 will allow to develop specific markers to monitor the behavior of PGPR populations over time in the rhizosphere and to verify the safety of using the strain as bio-inoculant.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402. doi:10.1093/nar/25.17.3389

Andrade ACS, Queiroz MH, Hermes RAL, Oliveira VL (2000) Mycorrhizal status of some plants of the *Araucaria* forest and the Atlantic rainforest in Santa Catarina, Brazil. Mycorrhiza 10:131–136. doi:10.1007/s005720000070

Bashan Y, Holguin G, Luz E (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can J Microbiol 577:521–577. doi:10.1139/W04-035

Bashan Y, Kamnev AA, de-Bashan LE (2013a) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fertil Soils 49:465–479. doi:10.1007/s00374-012-0737-7

Bashan Y, Kamnev A, de-Bashan L (2013b) A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. Biol Fertil Soils 49:1–2. doi:10.1007/s00374-012-0756-4

Bergottini V, Filippidou S, Junier T, Johnson S, Chain P, Otegui M, Zapata P, Junier P (2015) Genome sequence of *Kosakonia radicincitans* strain YD4, a plant growth-promoting rhizobacteria isolated from yerba mate (*Ilex paraguariensis* St. Hill.). Genome Announc

Bertrand A, Prévost D, Bigras FJ, Castonguay Y (2007) Elevated atmospheric CO_2 and strain of rhizobium alter freezing tolerance and cold-induced molecular changes in alfalfa (*Medicago sativa*). Ann Bot 99:275–284. doi:10.1093/aob/mcl254

Brady C, Cleenwerck I, Venter S, Coutinho T, De Vos P (2013) Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. Syst Appl Microbiol 36:309–319. doi:10.1016/j.syapm.2013.03.005

Bric JM, Bostock RM, Silverstone SE (1991) Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57:535–538

Brock A, Berger B, Mewis I, Ruppel S (2013) Impact of the PGPB *Enterobacter radicincitans* DSM 16656 on growth, glucosinolate profile, and immune responses of *Arabidopsis thaliana*. Microb Ecol 65:661–670. doi:10.1007/s00248-012-0146-3

Castric KF, Castric PA (1983) Method for rapid detection of cyanogenic bacteria. Appl Environ Microbiol 45:701–702

Cattelan AJ, Hartel PG, Fuhrmann JJ (1999) Screening for plant growth-promoting rhizobacteria to promote early soybean growth. Soil Sci Soc Am J 63:1670–1680

Chanway CP, Holl FB (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can J Bot 69:507–511

Collavino MM, Sansberro PA, Mroginski LA, Aguilar OM (2010) Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability

to promote *Phaseolus vulgaris* growth. Biol Fertil Soils 46:727–738. doi:10.1007/s00374-010-0480

Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959. doi:10.1128/AEM. 71.9.4951-4959.2005

Dobbelaere S, Croonenborghs A, Thys A (1999) Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil 212:155–164

Döbereiner J (1980) Forage grasses and grain crops. In: Bergersen F (ed) Methods for evaluating biological nitrogen fixation, New York J. pp 535–555

Eibl B, Fernandez RA, Kozarik JC, Lupi A, Montagnini F, Nozzi D (2000) Agroforestry systems with *Ilex paraguariensis* (American holly or yerba mate) and native timber trees on small farms in Misiones, Argentina. Agrofor Syst 48:1–8. doi:10.1023/A:1006299920574

Fages J, Arsac JF (1991) Sunflower inoculation with *Azospirillum* and other plant growth promoting rhizobacteria. Plant Soil 137:87–90. doi:10.1007/BF02187437

Fernandez R, Montagnini F, Hamilton H (1997) The influence of native tree species on soil chemistry in a subtropical humid forest region in Argentina. J Trop For Sci 10:188–196

Fox S, O'Hara G, Bräu L (2011) Enhanced nodulation and symbiotic effectiveness of *Medicago truncatula* when co-inoculated with *Pseudomonas fluorescens* WSM3457 and *Ensifer* (*Sinorhizobium*) *medicae* WSM419. Plant Soil 348:245–254. doi:10.1007/s11104-011-0959-8

Freitas JR, Germida JJ (1990) Plant growth promoting rhizobacteria for winter wheat. Can J Microbiol 36:265–272. doi:10.1139/m90-046

Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica (Cairo) 2012:1–15. doi:10.6064/2012/963401

Heck CI, De Mejia EG (2007) Yerba Mate Tea (*Ilex paraguariensis*): a comprehensive review on chemistry, health implications, and technological considerations. J Food Sci 72:R138–R151. doi:10.1111/j.1750-3841.2007.00535

Ilany T, Ashton M, Montagnini F, Martinez C (2010) Using agroforestry to improve soil fertility: effects of intercropping on *Ilex paraguariensis* (yerba mate) plantations with *Araucaria angustifolia*. Agrofor Syst 80:399–409. doi:10.1007/s10457-010-9317-8

James EK (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crop Res 65:197–209. doi:10.1016/S0378-4290(99)00087-8

Kertesz MA, Mirleau P (2004) The role of soil microbes in plant sulphur nutrition. J Exp Bot 55:1939–1945. doi:10.1093/Jxb/Erh176

Kloepper JW, Leong J, Teintze M, Schroth MN (1980) Enhanced plant-growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885–886. doi:10.1038/286885a0

Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol 7:39–44. doi:10.1016/0167-7799(89)90057-7

Liesack W, Weyland H, Stackebrandt E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb Ecol 21:191–198. doi:10.1007/Bf02539153

Mader P, Kaiser F, Adholeya A, Sing R, Uppal H, Sharma AK, Srivastava R, Sahai V, Aragno M, Wiemken A, Johri B, Fried P (2011) Inoculation of root microorganisms for sustainable wheat–rice and wheat–black gram rotations in India. Soil Biol Biochem 43:609–619. doi:10.1016/j.soilbio.2010.11.031

Madhaiyan M, Poonguzhali S, Lee JS, Saravanan VS, Lee KC, Santhanakrishnan P (2010) *Enterobacter arachidis* sp. nov., a plant-growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut. Int J Syst Evol Microbiol 60:1559–1564. doi:10.1099/ijs. 0.013664-0

Mehnaz S, Mirza MS, Haurat J, Bally R, Normand P, Bano A, Malik KA (2001) Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. Can J Microbiol 47:110–117. doi:10.1139/cjm-47-2-110

Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27:31–36. doi:10.1016/S0003-2670(00)88444-5

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic-relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel-electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165–172. doi:10.1007/Bf02529967

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. doi:10.1111/j.1574-6968.1999.tb13383

Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63:3367–3373

Peng G, Zhang W, Luo H, Xie H, Lai W, Tan Z (2009) *Enterobacter oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*. Int J Syst Evol Microbiol 59:1650–1655. doi:10.1099/ijs. 0.005967-0

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308–316. doi:10.1038/nchembio.164

Robin A, Vansuyt G, Hinsinger P, Meyer JM, Briat JF, Lemanceau P (2008) Iron dynamics in the rhizosphere: consequences for plant health and nutrition. Adv Agron 99:183–225. doi:10.1016/S0065-2113(08)00404-5

Rodrigues EP, Rodrigues LS, de Oliveira ALM, Baldani VLD, Teixeira KRD, Urquiaga S (2008) *Azospirillum amazonense* inoculation: effects on growth, yield and N_2 fixation of rice (*Oryza sativa* L.). Plant Soil 302:249–261. doi:10.1007/s11104-007-9476-1

Scholz-Seidel C, Ruppel S (1992) Nitrogenase- and phytohormone activities of *Pantoea agglomerans* in culture and their reflection in combination with wheat plants. Zentralbl Mikrobiol 147:319–328. doi:10.1016/S0232-4393(11)80395-1

Schwyn B, Neilands JB (1987) Universal chemical-assay for the detection and determination of siderophores. Anal Biochem 160:47–56. doi:10.1016/0003-2697(87)90612-9

Sharma SB, Sayyed RZ, Trivedi MH, Gobi TA (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus 2:587. doi:10.1186/2193-1801-2-587

Sing R, Adholeya A (2003) Interactions between arbuscular mycorrhizal fungi and plant-growth promoting rhizobacteria. Mycorrhiza News 15:16–17

Smyth EM, McCarthy J, Nevin R, Khan MR, Dow JM, O' Gara F, Doohan FM (2011) In vitro analyses are not reliable predictors of the plant growth promotion capability of bacteria; a *Pseudomonas fluorescens* strain that promotes the growth and yield of wheat. J Appl Microbiol 111:683–692. doi:10.1111/j.1365-2672.2011.05079

Stewart WDP, Fitzgera G, Burris RH (1967) In situ studies on N_2 fixation using acetylene reduction technique. Proc Natl Acad Sci U S A 58:2071–2078. doi:10.1073/pnas.58.5.2071

Tarnawski S (2008) Rhizosphere bacterial communities associated with *Lolium perenne*. University of Neuchâtel, PhD thesis

Tarnawski S, Hamelin J, Jossi M, Aragno M, Fromin N (2006) Phenotypic structure of *Pseudomonas* populations is altered under elevated pCO_2 in the rhizosphere of perennial grasses. Soil Biol Biochem 38:1193–1201. doi:10.1016/j.soilbio.2005.10.003

Witzel K, Gwinn-Giglio M, Nadendla S, Shefchek K, Ruppel S (2012) Genome sequence of *Enterobacter radicincitans* DSM16656^T, a plant growth-promoting endophyte. J Bacteriol 194:5469. doi:10.1128/JB.01193-12