

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

V. M. Bergottini<sup>1</sup> · M. B. Otegui<sup>2</sup> · D. A. Sosa<sup>3</sup> · P. D. Zapata<sup>2</sup> · M. Mulot<sup>4</sup> · M. Rebord<sup>1</sup> · J. Zoppi<sup>5</sup> · F. Wiss<sup>6</sup> · B. Benrey<sup>7</sup> · Pilar Junier<sup>1</sup>

**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<sub>2</sub> 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.

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✉ Pilar Junier  
pilar.junier@unine.ch

<sup>1</sup> Laboratory of Microbiology, University of Neuchâtel, Neuchâtel 2000, Switzerland

<sup>2</sup> Instituto de Biotecnología Misiones, Universidad Nacional de Misiones, Posadas 3380, Argentina

<sup>3</sup> Instituto Nacional de Tecnología Agropecuaria, EEA Cerro Azul, Cerro Azul 3313, Argentina

<sup>4</sup> Laboratory of Soil Biology, University of Neuchâtel, Neuchâtel 2000, Switzerland

<sup>5</sup> Department of Environmental Sciences, University of Basel, Basel 4056, Switzerland

<sup>6</sup> Instituto Nacional de Tecnología Agropecuaria, EEA Montecarlo, Montecarlo 3384, Argentina

<sup>7</sup> Laboratory of Evolutionary Entomology, University of Neuchâtel, Neuchâtel 2000, Switzerland

## 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 bio-fertilizers (Kloepper et al. 1980; Cattelan et al. 1999; Rodrigues et al. 2008; Tarnawski 2008), phyto-stimulators (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<sup>-1</sup> C, 3 g kg<sup>-1</sup> N, and 1.5 mg kg<sup>-1</sup> P with a pH of 6.3; in YD, the Ultisols contained 24 g kg<sup>-1</sup> C, 2 g kg<sup>-1</sup> N, and 2.9 mg kg<sup>-1</sup> P with an acidic pH of 4.5; and in YN, the Ultisols contained 734 g kg<sup>-1</sup> C, 4 g kg<sup>-1</sup> N, and 0.9 mg kg<sup>-1</sup> 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<sup>5</sup> 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 translucent halo after 72 h on National Botanical Research Institute's phosphate growth medium (NBRIP) agar plates with Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 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<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, AlPO<sub>4</sub> or FePO<sub>4</sub> at a concentration of 5 g L<sup>-1</sup>. 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<sup>8</sup> cells ml<sup>-1</sup>. 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 aryl-sulphatases was evaluated by the formation of a blue coloration around colonies on modified Angle agar plates with 40 mg L<sup>-1</sup> X-sulfate and 710 mg L<sup>-1</sup> Na<sub>2</sub>SO<sub>4</sub> 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<sup>3</sup>) with approximately 150 g of soil (Ultisol) or organic compost. The soil presented a pH of 5.17, 39,100 mg kg<sup>-1</sup> of C, 3700 mg kg<sup>-1</sup> of N, 13.94 of C/N, 3.85 mg kg<sup>-1</sup> of P, and 43 mg kg<sup>-1</sup> of K and the compost a pH of 5.07, 380,000 mg kg<sup>-1</sup> of C, 18,300 mg kg<sup>-1</sup> of N, 21.14 of C/N, 3033 mg kg<sup>-1</sup> of P, and 200 mg kg<sup>-1</sup> 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<sup>8</sup> cells ml<sup>-1</sup>. 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 homogeneous 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<sub>2</sub> 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<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 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<sub>2</sub> fixation was measured quantitatively for the most promising strains. *P. putida* YP2 and *Pseudomonas putida* YN3 displayed a significantly lower activity ( $P$  value  $\leq 0.05$ ), while the others did not differ significantly in their capability to fix N<sub>2</sub>. 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 aryl-sulphatases, 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 <sub>2</sub> fixation ( $\mu\text{mol}/\text{vial}\cdot\text{h}$ )	IAA ( $\mu\text{g ml}^{-1}/\text{OD600}$ )
Soil-degraded plantation	YD4	<i>Kosakonia radicincitans</i> (99 %)	+	42.1 a	24.4 bc
Productive plantation	YP3	<i>Rhizobium pusense</i> (99 %)	+	50.7 a	59.2 ab
Productive plantation	YP2	<i>Pseudomonas putida</i> (99 %)	–	1.5 b	5.0 d
Soil-degraded plantation	YD3	<i>Sphingobium yanoikuyae</i> (99 %)	–	nd	15.0 cd
Productive plantation	YP1	<i>Rhizobium cellulosilyticum</i> (99 %)	+	nd	10.7 d
Soil-degraded plantation	YD1	<i>Acinetobacter radioresistens</i> (99 %)	+	40.9 a	34.4 bc
Productive plantation	YP4	<i>Rhizobium pusense</i> (99 %)	+	nd	43.8 b
Soil-degraded plantation	YD2	<i>Kosakonia radicincitans</i> (99 %)	+	42.5 a	24.1 bc
Trees in the rainforest	YN4	<i>Acinetobacter radioresistens</i> (99 %)	+	41.1 a	12.3 d
Trees in the rainforest	YN2	<i>Ensifer adhaerens</i> (100 %)	–	47.9 a	11.5 d
Trees in the rainforest	YN1	<i>Ensifer adhaerens</i> (100 %)	–	nd	6.9 d
Trees in the rainforest	YN3	<i>Pseudomonas putida</i> (99 %)	–	2.8 b	3.7 d
	<i>A. brasilense</i>				
	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.

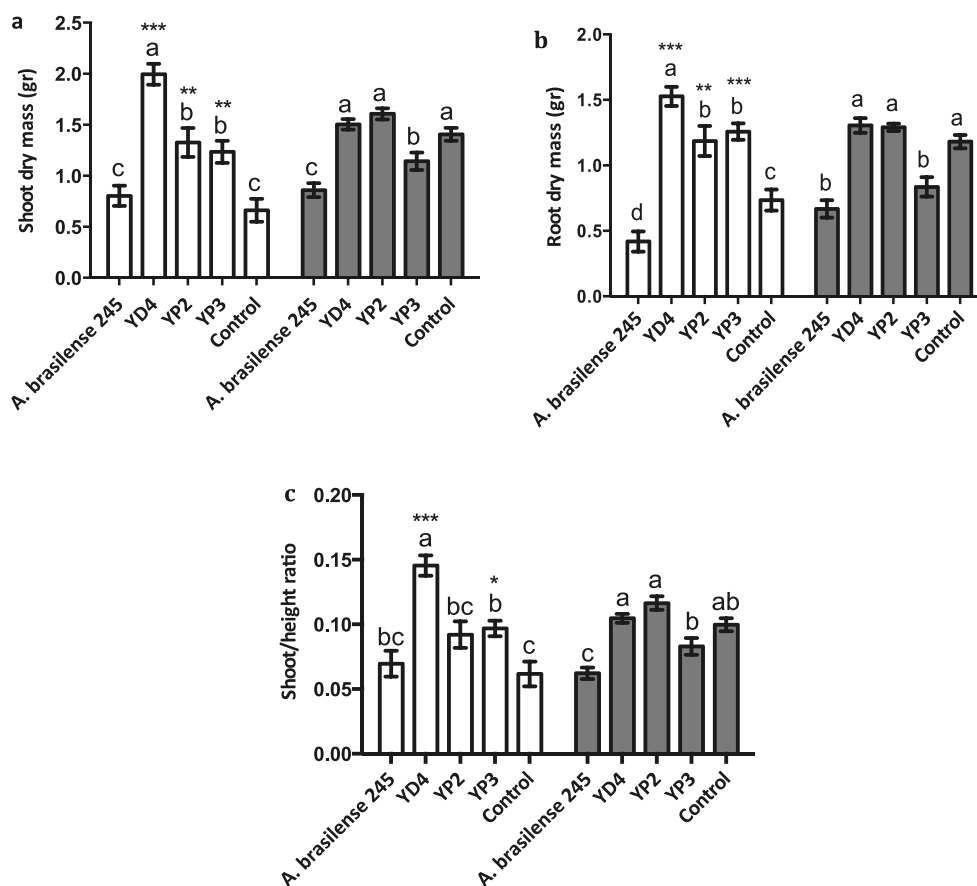
### Bio-inoculation assay

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  $\leq 0.05$ ). 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. radicinans* 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. radicinans* 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. radicinans* 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. radicinans* 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<sub>2</sub> 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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