Assessing the role of native Plant Growth-Promoting Rhizobacteria as bio-inoculants for Yerba Mate (*Ilex paraguariensis*)

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Veronica M. Bergottini

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Titre:

“Assessing the role of native Plant Growth-Promoting Rhizobacteria (PGPR) as bio-inoculants for Yerba Mate (*Ilex paraguariensis*)”

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Summary

Key words: *Ilex paraguariensis*, PGPR, *Kosakonia radicincitans*, bio-inoculant

Mots-clés: *Ilex paraguariensis*, PGPR, *Kosakonia radicincitans*, bio-inoculum

The accelerated process of soil degradation due to long-term inadequate agricultural practices in Misiones, the major productive region of Yerba Mate (*Ilex paraguariensis* St. Hill.) in Argentina, has led to an urgent need for research and development of more sustainable agricultural practices. Yerba Mate is an emblematic crop in southern South America due to its leaves are used to prepare an energizing beverage called “mate” consumed as an alternative to coffee. The main goal of this study was to evaluate the role of native plant growth promoting rhizobacteria (PGPR) as potential bio-inoculants for Yerba Mate seedlings.

This thesis has shown that biomass yields of Yerba Mate seedlings can be increased up to 183% through bio-inoculation with native PGPR strains. Interestingly, the biomass yield increase was obtained through bio-inoculation in a less fertile soil. *Kosakonia radicincitans* YD4 was the most effective PGPR strain in enhancing the growth of Yerba Mate seedlings. Bio-inoculation with this strain in soil led to higher yields than those obtained in compost, even though higher yields can be expected in the latter due its high fertility. These results suggested that this strain represents a promising candidate to evaluate its potential as bio-inoculant for this crop in low-productive plantations.

The whole genome of *K. radicincitans* YD4 was sequenced and annotated to analyze the genetic potential ability to colonize the rhizosphere, and if virulence-associated genes are present in this strain. The presence of genes for chemotaxis, adherence structures (fimbriae) and anti-microbial activities reflected the genetic potential of this strain to colonize and compete successfully in the rhizosphere. Regarding the safety of using YD4 as a bio-inoculant, two gene clusters (Type III and Type VI secretions systems) often associated to pathogenicity were identified. Comparative genomic analysis using closely related pathogenic and PGPR strains suggested that these gene clusters might confer adaptative advantages to YD4 to compete against other microbes and survive in the rhizosphere. This hypothesis has been also proposed for other beneficial plant associated bacteria possessing these gene clusters.

In order to evaluate the ability of the bio-inoculant YD4 to colonize the rhizosphere of Yerba Mate seedlings, a strain-specific real time PCR approach was designed. The dynamics of YD4 was monitored in the rhizosphere of inoculated seedlings during five weeks in

nursery. The plant growth-promoting effect of YD4 was confirmed by higher biomass yields and the introduced strain was detected in the rhizospheric soil until the end of the experiment (five weeks). These results suggested that the strain YD4 once inoculated, colonize and remain associated to the rhizosphere exerting its plant growth-promoting effect. Combining these results we can confirm that the isolate YD4 is a PGPR strain with a promising potential to be used as a bio-inoculant for Yerba Mate seedlings.

In the last chapter, the bacterial and fungal root-associated microbiome of Yerba Mate was analyzed for the first time using a pyrosequencing approach. This study aimed at the description of the enriched microbial taxa potentially involved in plant growth promotion inhabiting the roots of this crop. In addition, we analyzed if the agricultural historical management and location sites of Yerba Mate plantations have an effect on the alpha and beta diversity. Our results have shown that the bacterial genera *Burkholderia* and *Enterobacter* (containing well-known PGPR representatives) were enriched in Yerba Mate roots, whereas endomycorrhizal fungi were enriched particularly in one low-productive plantation with low soil P content. Yerba Mate trees might be exerting a selective pressure for mycorrhization in this type of plantations. The bacterial community composition, genetic diversity and phylogenetic diversity were significantly influenced by the geographical location of the plantations; whereas the fungal community composition and genetic diversity were significantly affected by the geographical location and soil pH. No effect of the historical agricultural management or location of the plantations was observed on the alpha diversity.

This thesis has contributed with an eco-friendly strategy to improve Yerba Mate growth in nursery, and highlighted the fact that better yields can be obtained thought bio-inoculation in less fertile soils. We are optimistic that this strategy could be exploited in field conditions in the future.

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Chapter 1: General Introduction

Research collaboration

The studies presented in this thesis are part of a research collaboration with two Argentinian institutions: Instituto de Biotecnología Misiones (Universidad Nacional de Misiones) and Instituto Nacional de Tecnología Agropecuaria (INTA). The main goal of this partnership is to approach the potential use of bio-inoculants to improve the growth of Yerba Mate seedlings (*Ilex paraguariensis* St. Hill.) in low-fertility soils in northeast Argentina. This collaboration allowed to combine the long tradition of the Laboratory of Microbiology (UniNe) in studying plant-bacteria interactions with the experience of Yerba Mate cultivation of our partners in Argentina.

1.1. Yerba Mate: the studied plant

The source of mate beverage

Ilex paraguariensis Saint Hilaire (Yerba Mate), a tree of the family Aquifoliaceae (Figure 1), is native to the Atlantic Forest in southern South America. The area of cultivation of this crop corresponds to the natural area of distribution of Yerba Mate (Grondona 1954) (Figure 2). This tree is a very valuable regional crop because its leaves are processed into a traditional beverage called mate, widely consumed in Argentina, Brazil, Paraguay, and Uruguay. Mate beverage is highly appreciated by millions of South Americans due to its stimulant effects and it is consumed as an alternative to coffee. Besides being a central nervous stimulant, mate presents high content of antioxidants and many benefits for human health (Heck and De Mejia 2007). In recent years, Yerba Mate has rapidly reached new markets where it is appreciated either as tea or as an ingredient in formulated foods or dietary supplements (Heck and De Mejia 2007). The preparation of mate beverage consists in filling a gourd with Yerba Mate tea where hot or cold water is poured and consumed by using a metal straw (Figure 3).

In the past, Yerba Mate was a highly appreciated tree in the indigenous Guarani communities due to its medicinal and stimulant effects. Then, during the Spanish colonization, the Jesuits discovered the potential of this tree, decided to domesticate the species and settled the first plantations (URL: http://en.wikipedia.org/wiki/History_of_yerba_mate, accessed the 1.4.2015). Nowadays, mate beverage represents an important cultural heritage and is part of the national identity of Argentina, Paraguay and Uruguay.

Yerba Mate cultivation

Principally cultivated as monoculture, Yerba Mate is managed as a shrub and the leaves are evergreen. As other species of the genus *Ilex*, Yerba Mate presents seed dormancy (Hu et al. 1979) and a process of stratification with soil in seedbeds is mandatory for germination.

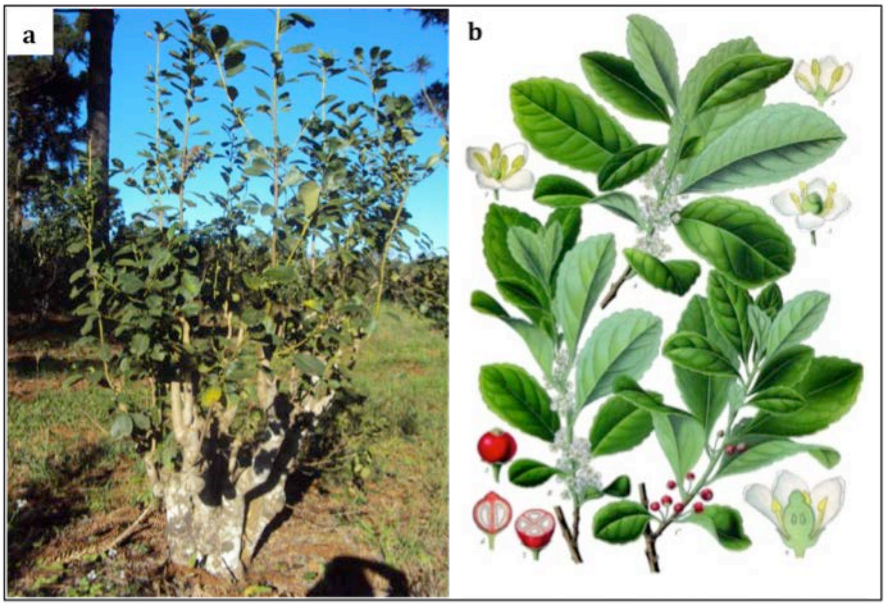

Figure 1. (a) A Yerba Mate tree in a plantation in Misiones, Argentina (Photo: V. Bergottini); (b) Illustration of *Ilex paraguariensis* St. Hill. (URL: http://es.wikipedia.org/wiki/Ilex_paraguariensis, accesed the 1.4.2015)

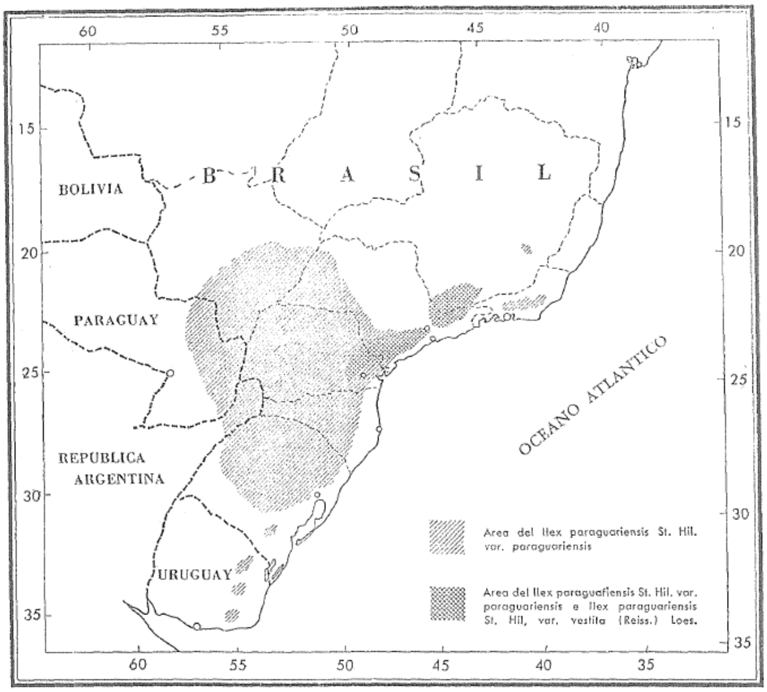

Figure 2. The area of distribution of Yerba Mate in southern South America (Grondona 1954) The area of production corresponds to its natural habitat.

Figure 3. Mate beverage.

The sowing in the seedbeds is performed between the months of March to May and it is important to control the sanitary and humidity conditions of the soil for a successful germination (Burtnik 2003). Seeds germination begins approximately after 100 days and can extend to one year after sowing (Burtnik 2003). During this period, seedlings are susceptible to be affected by damping-off caused by a complex of soil fungi and protists (*Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp., *Rhizopus* sp., *Phytophtora* sp.) (Burtnik 2003). After the germination (with a rate around 15%), the seedlings are transferred into individual pots in nursery, in which growth conditions are optimized to obtain good quality seedlings (Burtnik 2003). Seedlings are cultivated in pots with a rich substrate (organic compost, fertile soil from the rainforest, etc.) under controlled conditions of humidity, light and temperature (Burtnik 2003). Then, the growth is conducted under more natural conditions until seedlings are finally able to grow in open air conditions with minimal requirements of control (Burtnik 2003). The time needed from the germination in seedbeds until the seedlings are ready for the field transplantation is about eight months, and this takes place between the months of April and September (Burtnik 2003).

Yerba Mate requires high humidity levels and an annual precipitation of 1.000 mm well distributed throughout the year (Burtnik 2003). The preferred soil types for cultivation are the Ultisols and Oxisols, however, soil erosion and exhaustion occurs after few years of crop production (Moscatelli and Pazos 2000), and soils become low in organic matter, nitrogen and phosphorus content (Fernandez et al. 1997). The principal reasons for the decline in productivity are the age of trees and soil depletion due to inadequate agricultural management practices (e.g. 80-year-old plantations in continuous monoculture) (INYM-INTA 2008). Due to the current state of degradation, more sustainable practices have been recommended in Yerba Mate agricultural systems, i.e. the implementation of no-tillage agriculture (with the use of herbicides) and the introduction of covers crops (Burtnik 2003, Prat Kricun 2011) or native trees in mixed plantations (Eibl et al. 2000).

Current situation of Yerba Mate production

Argentina is the principal global producer of Yerba Mate with a total of 220.047, 37 hectares in the Northeast region of the country (URL: www.inym.org.ar/inym/imagenes/Estadisticas/Estractos_por_has.pdf, accessed the 1.3.2015). From these, 90% of the plantations are concentrated in Misiones province and the rest in the province of Corrientes (accessed the 1.3.2015 URL: www.inym.org.ar/inym/imagenes/Estadisticas/sup%20cultivada%20depa.pdf).

In Misiones this crop is an important socio-economic activity and many efforts have been conducted to improve the productivity principally in soil-degraded areas. Currently, Yerba Mate plantations are in a process of rapid degradation (INYM-INTA 2008). Although several approaches have been evaluated to improve productivity and soil quality in Yerba Mate plantations (Sosa 1992, Eibl et al. 2000, Day et al. 2011), the role of native microbes associated with this crop as a more sustainable strategy to improve growth and health of Yerba Mate has not yet been explored.

1.2. The rhizosphere

Definition

Plants obtain their resources for growth from soil, in which fungi, protozoa, and algae are also competing for resources. Bacteria are responsible for different functions related to soil fertility and plant health such as nutrient cycling, organic matter formation and decomposition, soil structure, and plant growth promotion (Kennedy 1999). In agroecosystems bacterial populations and their activities are influenced by the root system and the soil environment (Kennedy 1999). In soil there is a zone of interaction between plants and microbes considered as a hot spot due to the high microbial activity (reviewed in Hrynkiewicz and Baum 2012). This zone is the rhizosphere and it is defined as the volume of soil under the influence of the root (Hiltner 1904, Darrah 1991). This definition could be expanded to the rhizoplane (root surface), and the endorhizosphere (root tissues colonized by bacteria) (Gobat et al. 2004).

In the rhizosphere both, plant and microbes, have a reciprocal influence. The root influences the rhizosphere through many activities. One of them is the rhizodeposition, defined as the release of different types of compounds by the root (Figure 4) (Lynch and Whipps 1990). Among the rhizodeposition compounds, exudates (sugars, amino acids, organic acids, flavonoids, hormones, vitamins, etc.) are considered as the most important (Gobbat et al. 2004). In the rhizosphere the microorganisms benefit from these exudates and colonize the plant roots (Goddard et al. 2001). Others activities, including water and nutrients uptake, and root respiration, also affect the rhizosphere environment and consequently microbial activity (Marschner et al. 1997, Ghiglione et al. 2000). On the other hand, bacteria have also a marked influence on the plant in the rhizosphere. Their interaction with plants may be beneficial or harmful (Whipps 2001).

Plant Growth-Promoting Rhizobacteria (PGPR)

The bacteria that colonize successfully the rhizosphere are named rhizobacteria. There is a particular group of rhizobacteria that has a positive impact on plant growth and health collectively known as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1978). It is generally accepted that a rhizobacteria strain is considered as a PGPR when it can at least fulfill these two criteria: plant growth stimulation or biocontrol and rhizosphere colonization (Bhattacharyya and Jha 2012). The mechanisms of plant growth promotion can be direct or indirect. Direct stimulation occurs when bacteria function as a plant growth regulator, including changes in the concentration of plant hormones (Spaepen and Vanderleyden 2011), the facilitation of nutrients by biological N_2 fixation (Rodrigues et al. 2008), solubilization of inorganic phosphates (Mamta et al. 2010), mineralization of organic phosphates (Tarafdar and Claassen 1988), and production of siderophores (Kloepper et al. 1980). Indirect stimulation involves a variety of mechanisms that inhibit the effects of phytophatogens on plant growth, including antibiosis against phytopathogens (Compant et al. 2005), triggering the systemic resistance in plants (Pieterse et al. 1996), and creation of iron-limiting conditions through the production of siderophores controlling the proliferation of phytopathogens (Kloepper et al. 1980). Based on their mechanisms of action, PGPRs can be categorized into three general forms such as biofertilizers, phytostimulators and biopesticides (Bhattacharyya and Jha 2012) (Table 1).

The role of PGPR in agriculture

The plant growth promoting effects of PGPR have been demonstrated in different agronomic important crops in *in vitro* assays, in greenhouse conditions, and in some cases, in field trials (Utkhede et al. 1999, Dobbelaere et al. 2001). The effect observed in plant growth varies greatly. Examples include an increase in germination rates, higher biomass, better yields, higher leaf area and chlorophyll content, tolerance to drought, disease resistance conferred to the plant, and many others (Lucy et al. 2004). However, variability and inconsistency of the results obtained in the laboratory and field trials could be explained in part by the soil complexity and climatic variability that has a large impact on the effectiveness of PGPR (Lucy et al. 2004). In addition, the successful competitive adaptation of PGPR to the rhizosphere environment also depends on their rhizosphere competence (Goddard et al. 2001).

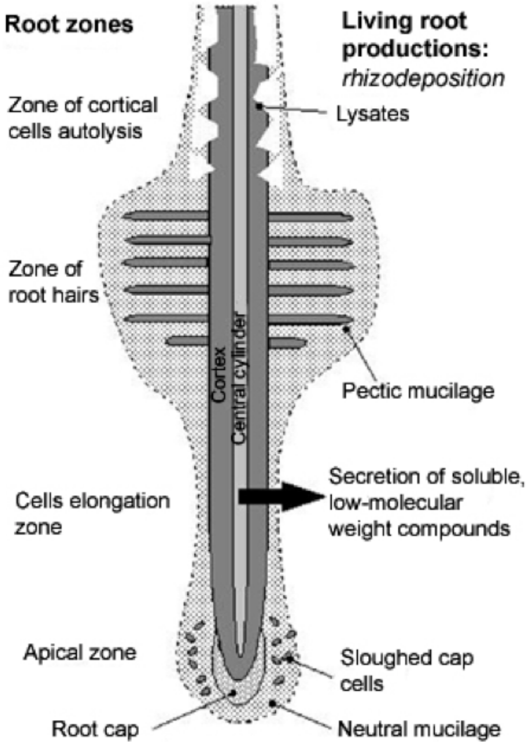

Figure 4. Diagram of a root and the rhizodeposition compounds released in the root zone (Source: Aragno and Tarnawski 2002)

Table 1. Categorization of PGPR and their mechanisms of action (Source: Bhattacharyya and Jha 2012)

PGPR forms	Definition	Mechanism of action	References
Biofertilizer	A substance that contains living microorganisms which, when applied on the seed, plant surface or soil, colonizes the rhizosphere and promote plant growth through increased supply of primary nutrients for the host plant	Biological nitrogen fixation Solubilization of insoluble phosphorus	Vessey (2003) Somers et al. (2004)
Phytostimulator	Microorganism, with the ability to produce phytohormones such as indole acetic acid, gibberellic acid, cytokinins and ethylene	Production of phytohormones	Lugtenberg et al. (2002) Somers et al. (2004)
Biopesticide	Microorganism that promote plant growth by controlling phytopathogenic agents	Production of antibiotics, siderophores, HCN Production of hydrolytic enzymes Induced systemic resistance	Vessey (2003) Somers et al. (2004) Chandler et al. (2008)

In the last decades, many efforts have been made in order to have a broader understanding of the traits involved in rhizosphere competence (Barret et al 2011). Some of the bacterial traits involved in rhizosphere competence are motility/chemotaxis, attachment (adhesins, pilli, fimbriae), growth, stress resistance, and production of secondary metabolites (Barret et al 2011).

Genetic basis of plant growth-promotion traits

Despite the fact that many plant growth-promoting mechanisms have been widely studied the knowledge about the genetic basis of many plant growth-promoting traits is still limited. In a recent study the distribution of some well-known genes involved in different plant growth promoting activities have been analyzed in PGPR strains and related *Proteobacteria,* for instance the operon *nifQBALFMZWVSUXNEYTKDHJ* for dinitrogen fixation i.e. in *Burkholderia* species, the pyrroloquinoline quinone operon *pqqBCDEFG* involved in the production of gluconic acid as mechanism of phosphate solubilization, the indole-3-pyruvate decarboxylase gene *ipdC* involved in the synthesis of indole acetic acid in *Azospirillum brasilense*, the anti-microbial encoding genes *hcnABC* for the synthesis of hydrogen cyanide and *phlACBD* for the synthesis of the 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens,* and some others genes (Bruto et al. 2014)

Currently, the whole genomes of many PGPR and plant growth promoting endophyte strains are available in public databases, allowing comparative analysis of functions involved in plant-bacteria interactions (Wisniewski-Dyé et al. 2012, Shen et al. 2013, Gupta et al. 2014). Rhizosphere competence traits are also evaluated to reveal functions for an effective rhizosphere colonization including chemotaxis towards roots exudates, adhesion mechanisms (pili, fimbriae, polysaccharides, adhesins) and metabolization of roots exudates (Taghavi et al. 2010, Liu et al. 2013). In addition, the available genomes of many plant and human microbial pathogens allow to perform comparative analysis of virulence-associated genes for instance the secretion systems Type III (Tampakaki 2014) and Type VI (Shrivastava and Mande 2008) responsible for the pathogenicity of some species. These analyses are important for a bacterial inoculant to confirm that it does not represent a sanitary risk for the human beings and for the environment.

1.3. Biodiversity

Biodiversity can be defined as the variety of different organisms in a system. This can be analyzed at different levels. The diversity on a local habitat is the alpha diversity, the variation of species composition among habitats is the beta diversity, and the diversity in along a landscape of different habitats is the gamma diversity (Whittaker 1972).

Diversity consists of two components: species richness (the number of different species in a community) and evenness (how individuals are distributed among these species) (Margalef 1968). Since there is still no consensus for the concept of species in prokaryotes, the term operational taxonomic unit (OTU) is used to indicate those groups that cluster together above a pre-defined similarity threshold (e.g. 97% for the 16S rRNA gene). The diversity can be measured by using diversity indices or by applying rarefactions curves. The latter represents the number of species/OTUs obtained as a function of the sample size. If the curve does not reach the asymptote indicates that a fraction of the diversity remains to be analyzed, while if the curve reaches the asymptote, it will provide an estimation of the species/OTUs richness in the community.

Nowadays the microbial diversity can be approached by cultivation-independent methods. Analysis of biochemical markers as phospholipid fatty acid analysis (PFLA) is one of these approaches (Zelles et al. 1995), however it is not useful to identify microorganisms at the species level (Haack et al. 1994). Alternatively (and more widely used) the methods based on the analysis of nucleic acids extracted directly from soil allow to analyze the microbial structure and functioning in soils. Among the more widely genetic markers used, the 16S rRNA gene and the internal transcribed spacer (ITS) region are used to assess the bacterial and the fungal communities, respectively. Often, in order to have a first picture of the microbial communities in soil molecular fingerprinting techniques are used as routine in many laboratories. Among these techniques, denaturing gradient gel electrophoresis (DGGE) allows to evaluate the microbial communities in environmental samples (Muyzer et al. 1993) with the possibility to analyze bands of interest by cloning and sequencing. Nowadays, Next Generation Sequencing (NGS) is used to examine the microbial composition and diversity of complex microbial communities. The advantage of these technologies is that they allow to study those minority taxa, called rare biosphere (Sogin et al. 2010).

The rhizosphere microbiome

There is an increasing interest in elucidating the root associated-microbiome of different plant species (Uroz et al. 2010, Debenport et al. 2015) since these microbiota play a major role in plant growth and plant protection. In a recent study, culture-independent microbial community profiling was used to analyze the enriched diazotrophic bacteria associated to the rhizosphere and roots of sugarcane in order to guide the isolation and selection of putative bacterial inoculants for this crop (Paungfoo-Lonhienne et al. 2014).

The rhizosphere is a particular microhabitat in which the microbiota is influenced by many factors. Soil properties and plant species have a major influence in the structure and function of microbial communities (Berg and Smalla 2009). Some studies have demonstrated the selective pressure that can exert the plant on the soil microbiota reservoir (Gottel et al. 2011, Lundberg et al. 2012). In some cases the selection is exerted on some functional groups of relevance for the plant. For instance, the perennial grass *Molinia coerulea* grown in low nitrogen prairies resulted in the selection of a few specific rhizosphere diazotrophic populations (Hamelin et al. 2002). Besides these biotic influences, there are many other abiotic factors such as soil physicochemical properties, temperature, water content as well as anthropogenic factors such as the agricultural management practices of the fields contributing to shape the microbial community composition and diversity in soils (Nunes da Rocha 2009). Among these factors, soil pH has been shown to have a markedly effect on the bacterial community composition at a continental scale (Lauber et al. 2009) and even at a field scale (Rousk et al. 2010).

Root-associated microbiome of Yerba Mate

Some studies have explored the microbial communities (potentially involved in plant growth promotion) associated with Yerba Mate. Collavino et al. (2010) isolated from Yerba Mate rhizosphere phosphate-solubilizing bacteria assigned to the genera *Enterobacter*, *Pantoea*, *Pseudomonas*, *Acinetobacter*, *Burkholderia*, and *Exiguobacterium*. Some of these isolates promoted plant growth and increased phosphorus and nitrogen contents of leaves in *Phaseolus vulgaris* (Collavino et al. 2010). Although these isolates were native to Yerba Mate, they were not tested as bio-inoculants in this crop. In another study, a diversity analysis of the endophytic bacterial populations in Yerba Mate leaves and stems showed that the most dominant group was the order *Actinomycetales*, while *Curtobacterium* and *Microbacterium* were the most dominant genera (Perez et al. 2012). In this study the following genera were also reported as Yerba Mate endophytes: *Curtobacterium, Herbiconiux, Leucobacter, Mycobacterium, Microbacterium, Aeromicrobium, Cellulosimicrobium, Kineococcus, Methylobacterium, Agrobacterium, Rhizobium, Roseomona, Bacillus, Paenibacillus, Staphylococcus, Sphingomonas* and *Brevundimonas* (Perez et al. 2012). Regarding the fungal root-associated microbiome very few is known about the taxa and functional groups that could be involved in plant growth promotion. A previous study reported that Yerba Mate seedlings presented high rates of micorrhization in nursery conditions (Gaiad and Sidney Lopes 1986). Since this study there were no other research conducted in this area.

Since there are very few studies about the root-associated microbiome of this crop, a deeper analysis of the bacterial and fungal communities will allow to guide the selection and isolation of potential beneficial microbes in future studies.

1.4. Research objectives

Many approaches have been evaluated to improve Yerba Mate growth in low-fertility soils. However the potential use of bio-inoculants to enhance crop yields has not been assessed so far. Taking into consideration several successful cases in which bio-inoculants enhanced plant growth and health, we hypothesized that Yerba Mate rhizosphere could represent a reservoir for selecting potential PGPR strains adapted to endemic soil conditions and that will have a positive effect on growth, particularly in poor soils. Therefore, the main goal of this study was to evaluate the role of native plant growth promoting rhizobacteria (PGPR) as potential bio-inoculants for Yerba Mate seedlings in less fertile soils.

In chapter 2 the aims were to isolate, characterize and select rhizobacteria strains from Yerba Mate to assess their role as PGPR in Yerba Mate seedlings in nursery. In order to select the most promising PGPR strain, the performance of native versus non-native PGPR strains in soils with different nutritional status were compared.

In chapter 3 the aims were to analyze in the selected PGPR strain the genetic basis for inferring: i) its potential ability to colonize successfully the rhizosphere and, ii) the presence of virulence-associated genes to infer a potential pathogenic lifestyle.

In chapter 4 the aim was to experimentally approach the rhizosphere colonization ability and persistence of the selected PGPR strain using a quantitative real-time PCR approach.

In chapter 5 the aims were to explore the root-associated microbiome of this crop using high-throughput pyrosequencing in order i) to analyze the factors that determine the bacterial and fungal community composition and the genetic diversity and, ii) to identify potential beneficial microbes for further biotechnological applications.

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Chapter 2: Assessing the role of native PGPR strains as bio-inoculants for Yerba Mate

This chapter is based on the published article:
VM Bergottini, MB Otegui, DA Sosa, PD Zapata, M Mulot, M Rebord, J Zopfi, F Wiss, B Benrey, P Junier (*in press*). Bio-inoculation of Yerba Mate seedlings (*Ilex paraguariensis* St. Hill.) with native plant growth promoting rhizobacteria: a sustainable alternative to improve crop yield. Biology and Fertility of Soils.
Additional experiments and supplementary information not included in the article are presented as well.

2.1. Summary

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 five-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.

2.2. Introduction

Yerba Mate (*Ilex paraguariensis* St. Hill.), a species of the family Aquifoliaceae, is a native tree of northeastern Argentina, Paraguay, South Brazil and part of Uruguay (Grondona 1954). 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 its stimulant effects. Moreover, Yerba Mate popularity is expanding to new markets including 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 hectares) concentrated in Misiones province (www.inym.org.ar/inym/imagenes/Estadisticas/Estractos_por_has.pdf, accessed the 10.12.2014). About 50% of these plantations are currently in a process of degradation (INYM-INTA 2008). 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) (INYM-INTA 2008). In Misiones, Yerba Mate is typically cultivated in Ultisols and Oxisols, however after a few years of crop production, fertility decreases substantially (Moscatelli and Pazos 2000). 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 (INYM-INTA 2008).

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. PGPR can behave as bio-fertilizers when they facilitate the uptake of nutrients through the fixation of N_2 (Rodrigues et al. 2008), solubilization of inorganic phosphates (Mamta et al. 2010), or production of siderophores (Kloepper et al. 1980) and sulphatases (Tarnawski et al. 2008). PGPR can be considered as phytostimulators when they synthesize phytohormones (Spaepen and Vanderleyden 2011). Finally, PGPR can favor plant health through the control of pathogens by producing antibiotics (Compant et al. 2005) or by inducing systemic resistance (Pieterse et al. 1996).

The rhizosphere and roots of several crops are relevant microniches for the isolation of native PGPR strains adapted to endemic soils 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.

2.3. Material and methods

Isolation of rhizobacteria

Three types of environments were selected to collect Yerba Mate roots: a productive plantation (YP) (20 years old), a soil-degraded plantation (YD) (35 years old) and, Yerba Mate trees (YN) (40 years old) from the rainforest. Sampling was carried out in winter in July 2010. All the sites were located in San Vicente (175 m.a.s.l), Misiones province (YP: 26°54’58.03’’S 54°25’40.47’’W; YD: 27°02’58.10’’S 54°30’29.12’’W; YN: 20°55’35.88’’S 54°25’57.79’’W). In YP, the Ultisols contained 32 g kg^{-1} C, 3 g kg^{-1} N, 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, 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, 0.9 mg kg^{-1} P with a pH of 5.8. Five plants were selected in each environment to collect 50 grams of active roots (depth up to 20 cm). Roots were transported to the laboratory (at 4 °C).

For bacterial isolation, ten grams 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 minutes. The soil suspension was filtered (sterile filter paper), diluted ten-fold 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 x 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) according to the manufacturer's instructions. DNA was quantified with a Qubit® dsDNA BR Assay Kit with a Qubit®2.0 Fluorometer (Invitrogen Ltd., UK) according to the manufacturer's instructions. 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 amongst 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

Phosphate solubilization assay

The capacity of the isolates to solubilize inorganic phosphorus was evaluated on NBRIP agar plates with $Ca_3(PO_4)_2$ as sole phosphate source (Nautiyal 1999). Fresh cultures were inoculated on NBRIP plates and incubated at 30 °C. Solubilizing strains were determined by the formation of a surrounding translucid halo after 72 h. This trait was verified on solidified medium after two consecutive transfers of the cultures. After the bio-inoculation assay, phosphate solubilization was verified in the selected strains 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 supernantant was determined by the molybdenum blue method (Murphy and Riley 1962). *Enterobacter cloacae* strain Neu 1027 and *Azotobacter chroococcum* strain BNM 363 were used as positive and negative controls, respectively.

Siderophores production assay

The ability of the isolates to synthesize siderophores was evaluated in blue-agar CAS medium (Schwyn and Neilands 1987). Fresh cultures were inoculated on this medium and incubated at 30 °C. A positive result was evidenced by the formation of an orange halo around the colonies after 48 h of incubation. *Pseudomonas fluorescens* strain Neu 1043 and *A. chroococcum* strain Neu 1159 were used as positive and negative controls, respectively.

N_2 fixation assay

The acetylene reduction assay (ARA) (Stewart et al. 1967) was performed to quantify the ability of the isolates to fix N_2. The nitrogenase activity was analyzed in 20 mL vials containing 8 mL of semi-solid LG medium. Five ml of a bacterial suspension adjusted to an abundance 10^8 cells mL^{-1} was inoculated in the semi-solid media and incubated at 30 °C for 48 h. Afterwards, the vials were sealed hermetically with a rubber stopper and 10% of the atmosphere was substituted by acetylene. The cultures were incubated for 2 h at 30 °C in darkness prior to measurement. The produced ethylene was measured by injecting 100 µL of the headspace into a SRI Instruments 8610C gas chromatograph equipped with a HayeSep T column (at 70 °C) and a flame ionization detector (FID at 150 °C). In the N_2 fixation assay *Azospirillum brasilense* strain 245 and *Escherichia coli* strain Neu 1006 were included as positive and negative controls, respectively.

Aryl-sulphatases production assay

The synthesis of aryl-sulphatases was evaluated on modified Angle agar plates with 40 mg L^{-1} X-sulfate and 710 mg L^{-1} Na2SO4 as sulfur sources (Kertesz and Mirleau 2004). A blue coloration around the colonies confirmed the degradation of X-sulfate by aryl-sulphatases. An un-indentified rhizosphere isolate (Jossi 2008) and *Enterobacter aerogenes* strain Neu 1036 were used as positive and negative controls, respectively.

Auxin-like production assay

The ability of the isolates to synthesize 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. *A. brasilense* strain 245 and *P. fluorescens* strain Neu 1043 were used as positive and negative controls, respectively.

Hydrogen cyanide assay

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). *P. fluorescens* strain Neu 1201 and *P. fluorescens* strain Neu 1027 were used as positive and negative controls, respectively.

Antagonisms assay

The isolates were also tested for their ability to inhibit the growth of fungi and protists responsible for damping-off attack, since this is often encountered in plantations and in nursery. A dual culture assay on PDA was performed 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* or *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. *P. fluorescens* strain CHAO and *E. coli* strain Neu 1006 were used as positive and negative controls, respectively.

Plant inoculation assay

Three selected isolates 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. Four inoculants treatment (strains selected as putative plant growth promoters and *A. brasilense* strain 245 as model PGPR) were tested in two types of substrates, soil and organic compost. Yerba Mate seedlings with the most homogenous phenotype were selected from the seedbed to be transplanted into pots (200 cm3) with approximately 150 g of soil (Ultisol) or organic compost. The soil presented a pH of 5.17, 39100 mg kg^{-1} of C, 3700 mg kg^{-1} of N, 13.94 of C/N, 3.85 mg kg^{-1} of P, 43 mg kg^{-1} of K; and the compost a pH of 5.07, 380000 mg kg^{-1} of C, 18300 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, we irrigated with 5 mL of a fresh bacterial suspension (in sterile water) 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 3 replicates of 10 plants per treatment. After 6 months post-inoculation plants were harvested to analyze shoot and root dry weight, height, and the concentration of macronutrients in shoots. As some Yerba Mate seedlings were not homogenous in their height, we normalized the shoot dry weight by seedlings 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 (Kjeldahl 1833), P by the vanadate/molybdate method (Chapman and Pratt 1962), K by flame photometry, Ca and Mg by EDTA titration (Bock 1962).

Statistical analyses

The mean of N_2-fixation rate and production of IAA-like compounds were compared by a one-way ANOVA and pairwise differences were tested using Tukey's post-hoc test ($P \leq 0.05$). The effect of bio-inoculants on plant biomass in each substrate was compared in the same way. 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.

2.4. 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). In order 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 aryl-sulphatases assay, but only *Sphingobium yanokuyae* YD3 was positive. Bio-control activities were also evaluated. None of the isolates showed the ability to produce hydrogen 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 (Figure 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% 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 (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, which induced a substantial increase in plant biomass, did not enhance the concentration of any of the measured macroelements (Table 2). In compost, bio-inoculants produced significant increases in the content of macronutrients (N, P, K, Ca) compared to controls. N concentration increased by 40% and 20% in plants inoculated with *P. putida* YP2 and *R. pusense* YP3, respectively, while P concentration increased by 40% with *A. brasilense* 245, *K.*

radicincitans YD4 and *P. putida* YP2. Bio-inoculation with *K. radicincitans* YD4 and *A. brasilense* 245 produced an increase by 63% and 33%, respectively, in K concentration. Finally, *P. putida* YP2 was the inoculant that increased the most Ca concentration (16%).

2.5. 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 biofertilization, phyto-stimulation or biocontrol, we favored 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 (Mäder et al. 2011). Interestingly, when biomass yields are compared between soil and compost, better yields were obtained in soil through the bio-inoculation with *K. radicincitans* YD4, although 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, Mäder et al. 2011). Even though 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 could be explained by a synergic combination of all 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 as suggested in literature (James 2000). Likewise, the ability to produce IAA-like compounds in stimulating root development and subsequently increases in water and nutrients 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).

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}/OD_{600})**
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 post-hoc test.

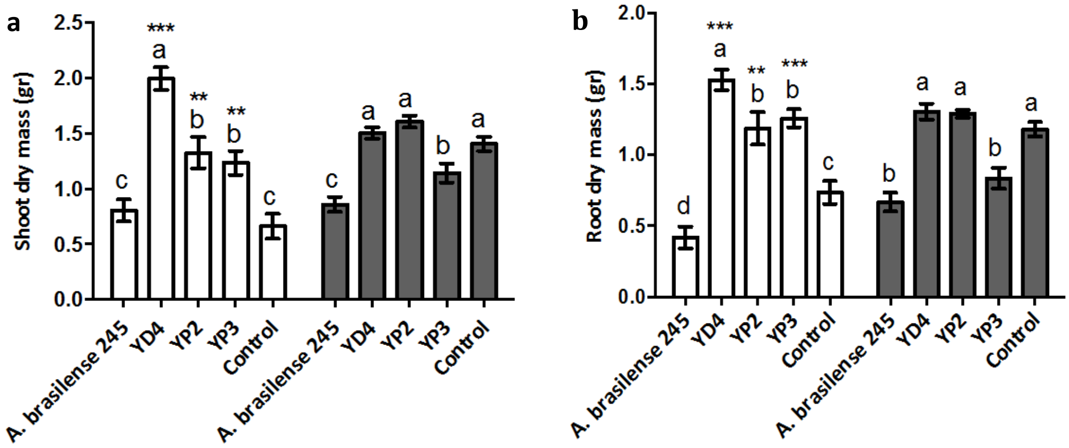

Figure 1. Effect of bio-inoculation with native rhizobacteria on the growth of Yerba Mate (*Ilex paraguariensis*) cultivated in soil or compost in nursery. Seedlings were inoculated with selected rhizospheric isolates from Yerba Mate plantations, *K. radicincitans* YD4, *P. putida* YP2 and *R. pusense* YP3; and with *A. brasilense* 245, strain used as bio-inoculants for several crops. Means of shoot dry mass (a) and root dry mass (b) 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 1-way ANOVA and Tukey as post hoc-test. Statistical significant differences in bio-inoculant's performances in comparison with non-inoculated controls are indicated by different letters (P ≤ 0.05). Statistical differences between bio-inoculants and the control conditions in soil are shown as: *** P ≤ 0.001, ** P ≤ 0.01 and * P ≤ 0.05.

Table 2. Content of nutrients measured in shoots of Yerba Mate seedlings inoculated with native PGPR isolates.

	Bio-inoculant	**Macronutrients in shoots (g kg^{-1})**				
		N	P	K	Ca	Mg
Soil*	YD4	11.7 ± 0.05	0.8 ± 0.01	5.1 ± 0.01	21.3 ± 0.40	6.3 ± 0.30
	YP2	7.6 ± 0.13	0.9 ± 0.01	4.9 ± 0.02	22.3 ± 0.09	6.1 ± 0.07
	YP3	9.1 ± 0.15	1.1 ± 0.02	5.5 ± 0.04	17.2 ± 0.53	6.1 ± 0.12
	Control	9 ± 0.10	0.9 ± 0.03	4.8 ± 0.05	20.2 ± 0.32	5.1 ± 0.14
Compost	*A. brasilense* 245	14.1 ± 0.23	1.4 ± 0.01	3.2 ± 0.03	20.8 ± 0.35	4.9 ± 0.11
	YD4	14.6 ± 0.38	1.4 ± 0.01	3.9 ± 0.05	23.6 ± 0.09	6.9 ± 0.10
	YP2	16.9 ± 0.12	1.4 ± 0.01	3.7 ± 0.05	25.8 ± 0.08	5.8 ± 0.10
	YP3	15 ± 0.02	1.1 ± 0.02	2.8 ± 0.04	23.3 ± 0.09	6.3 ± 0.16
	Control	12.1 ± 0.13	1 ± 0.01	2.4 ± 0.02	22.2 ± 0.09	5.6 ± 0.10

*Values for *A. brasilense* 245 in soil could not be obtained.

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. 2013). *K. radicincitans* YD4 initially displayed this activity but it was not detected when checked after the bio-inoculation assays were performed. This was the case even when different metal-phosphate sources were tested as suggested by Bashan et al. (2013). 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. 2013). 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 is one of the plant growth promoting mechanisms of *K. radicincitans* 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, Mäder 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 and macronutrient contents of inoculated Yerba Mate seedlings in soil and compost, respectively. 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 due to the high background of the microbial community in the samples. Therefore, complementing the current study with the analysis of the genome of YD4 will allow to verify the safety of using the strain as bio-inoculant and also to develop specific markers that can be used in future to monitor the behavior of PGPR populations over time in the rhizosphere.

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2.8. Appendix

Additional experiments

Four experiments, not included in the article, are described in this section. In some cases we faced technical issues, in others un-conclusive results. Anyway, these experiments were crucial to re-design our experimental approach that led to obtain a published work.

2.8.1. Bio-inoculation of Yerba Mate

During the selection process of putative PGPR strains, there are generally three stages of analysis in plants. Firstly, bio-inoculation is evaluated on seed germination or on the growth of young seedlings under gnobiotic conditions; then on plants grown under greenhouse conditions; and finally, in fields trials. We planned to follow these stages of analyses to have preliminary results in the laboratory before evaluating the PGPR strains as bio-inoculants in a broader scale (*in vivo* assays). Therefore, a first objective was to perform a plant bio-inoculation assay with the three selected PGPR strains in Yerba Mate seedlings cultivated in growth chambers.

Methods: cultivation of rudimentary embryos and cut pyrenes

Two methods were assessed in order to obtain Yerba Mate seedlings: *in vitro* culture of rudimentary embryos (Sansberro et al. 1998) and *in vitro* culture of cut pyrenes (recommended for *Ilex dumosa*) (Dolce et al. 2011). Yerba Mate, as others species of the genus *Ilex*, presents seed dormancy with rudimentary embryos (heart-shaped stage), which makes the germination process difficult *in vitro* (Hu et al. 1979). Yerba Mate seeds provided by INTA EEA-Cerro Azul (harvest 2012) were surface disinfected and excised under a laminar flow hood. Embryos and cut pyrenes were cultivated on quarter-strength Murashige and Skoog (MS) medium supplemented with 3% sucrose, 0.65% agar and 0.1 mg L^{-1} zeatin for 28 days in darkness at 27 ± 2°C. Germinated embryos were grown at 27 ± 2°C with a 14-h photoperiod (116 μmol m^{-2} s^{-1}) (Sansberro et al. 1998). The seedlings obtained were then transferred into Leonard Jars with MS medium without sucrose until inoculation.

Results and Discussion

The *in vitro* culture of immature embryos and cut pyrenes presented many technical difficulties, including abnormal development of seedlings and recurrent contamination (Figure 1-2), issue commonly observed in the micro propagation of Yerba Mate in bioreactors (Luna et al. 2013). Recently, it was suggested to use biocides to control contaminations of endophytic bacteria for the micro propagation of Yerba Mate (Luna et al. 2013). Since the *in vitro* embryos culture is known to be very laborious and time consuming, we expected to obtain better results with the *in vitro* culture of cut pyrenes. This approach recommended for *Ilex dumosa* is reported to be easier and more effective than the culture of embryos (1000 cut pyrenes per day can be cultured instead of 100 isolated embryos) (Dolce et al. 2011). However no seedlings were obtained due to recurrent contaminations (Figure 2). Therefore, the number of embryos that developed successfully was not enough to perform a meaningful bio-inoculation assay under laboratory conditions. Due to the difficulties to obtain Yerba Mate seedlings *in vitro,* the role of putative PGPR strains should be assessed in nursery. We consider that the strategy to select PGPR strains for this crop cannot follow the typical stages of analysis performed in other plant species. Our experience suggests that growth-chamber tests should not be approached. Therefore nursery assays should represent the starting point for testing a putative bio-inoculant for this crop.

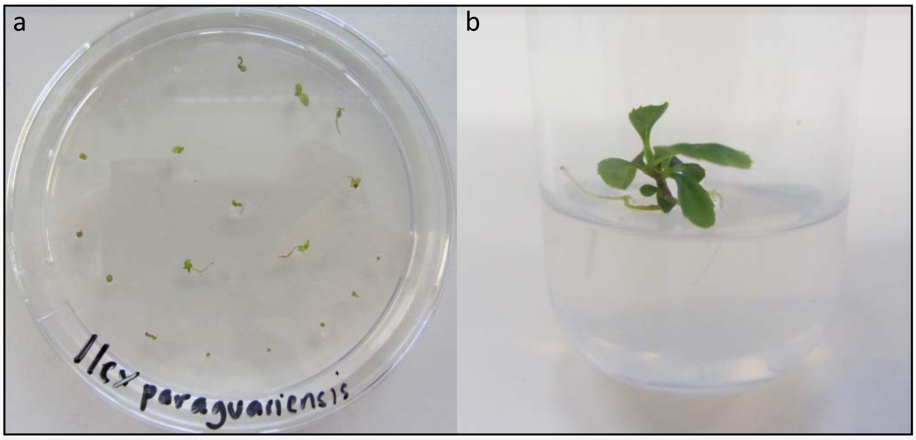

Figure 1. *In vitro* culture of rudimentary Yerba Mate embryos: (a) abnormal development of same seedlings and, (b) four month-old seedling successfully cultivated.

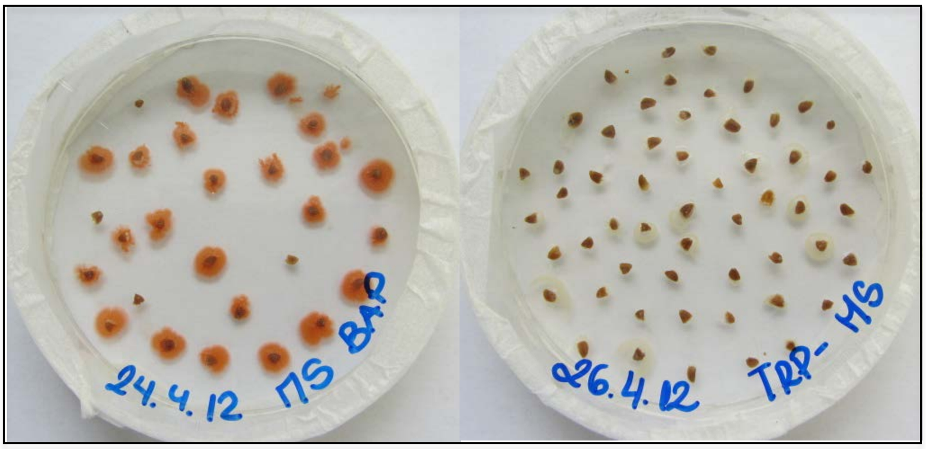

Figure 2. Contaminations in the *in vitro* culture of cut pyrenes

2.8.2. Bio-inoculation assay of Phaseolus vulgaris

In parallel to experiments with seedlings from *in vitro* culture, the three selected isolates were tested as bio-inoculants in *P. vulgaris* (bean) plants, used as a model due to its abundant shoot biomass and fast germination rate.

Methods

Bean seeds of a commercial variety were germinated in pots (75 cm^3) with sterile vermiculite in a climatic chamber at 28°C. Inoculation was performed on seven-day-old seedlings by irrigating roots with 3 ml of a bacterial suspension (in water) adjusted to a density of 10^8 cells mL^{-1}. Bio-inoculants were evaluated in plants irrigated with different concentrations of the nutritive solution MS (1X, 0.75X, 0.5X, 0.25X, 0.125X) to analyze the effect on plant growth at the different nutritional regimes. Plants grown at 28°C with a photoperiod of 12h were irrigated with the nutritive solution every 15 days and watered twice per week. After one month of cultivation, plants were harvested for the analysis of dry shoot mass and specific foliar area (SLA).

Results and discussion

Bio-inoculation with the native strains of Yerba Mate did not increase the dry shoot mass of plants at any concentration of the nutritive irrigation regime. However, a positive significant effect ($p \leq 0.05$) was observed in the specific foliar area (SLA) of plants inoculated with the strain *Pseudomonas putida* YP2 at the two lowest nutritive regimes (0.25X and 0.125X MS solution) (Figure 3). These results reflect the importance to inoculate plants with native PGPR strains rather than non-native strains as discussed previously in the article. In addition, the only positive effect was observed in plants irrigated with the less nutritive concentration regime, suggesting once again that a more pronounced plant growth promoting effect is observed under less fertile conditions. We concluded that the experiment with bean plants only gave us a small indication of the potential of one of our strains (YP2), suggesting that our native strains should be tested direct in Yerba Mate seedlings *in vivo* for having a first idea of their potential as bio-inoculants.

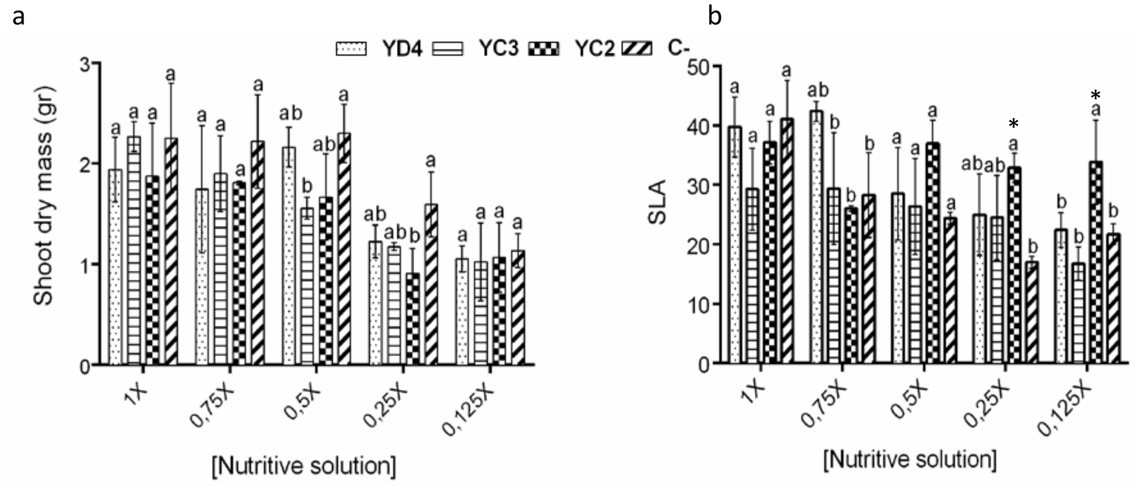

Figure 3. Effect of the bio-inoculation with native rhizobacteria on the growth of *Phaseolus vulgaris* cultivated under controlled conditions. (a) Shoot dry mass and, (b) Specific foliar area (SLA) of plants inoculated with YD4, YP3, YP2 and control plants after 30 days of cultivation under different concentrations of Murashige and Skoog solution (1X, 0.75X, 0.5X, 0.25X and 0.125X). Performances among bio-inoculants are compared within each concentration separately (1-way ANOVA and Tukey as post hoc-test). Statistically significant differences in the SLA among bio-inoculants and the control conditions are indicated as * ($p \leq 0.05$)

2.8.3. Traceability of the bio-inoculants in the in vivo assay

In addition to their plant growth promoting (and biocontrol) traits, inoculant microorganisms should be able to competitively survive and colonize the rhizosphere. This ability is called rhizosphere competence. We hypothesized that the plant growth promotion effect of an introduced PGPR strain depends on its ability to colonize successfully the rhizosphere. In this experiment our objective was to detect the presence of the introduced native PGPR strains in the rhizosphere and on roots of the Yerba Mate seedlings of the bio-inoculation assay conducted in nursery.

Methods

The presence of the introduced bacteria in the seedlings rhizosphere was analyzed by the fingerprinting technique Denaturing Gradient Gel Electrophoresis (DGGE). Two months post inoculation, roots of three plants per treatment were collected and washed with sterile water to perform DNA extraction with the FastDNA Spin Kit for Soil (MP Biomedicals, California). PCR amplification was performed on the V3 region of the 16S rRNA gene with the primers 338f (5’- ACTCCTACGGGAGGCAGCAG-3’) and 520r (5’-ATTACCGCGGCTGCTGG-3’) with a 40 bp GC-clamp on the forward primer (Muyzer et al. 1993). PCR products adjusted to 500 ng DNA per sample were analyzed by DGGE (8% acrylamide with a gradient of 30-60% of denaturants) during 4 h at 150V at 60 °C. The gel was stained in a solution of Sybr Gold 0.01% and visualized with a Multi-Analyst system (VWR, France). The ladder (L) consisted of the V3 region sequences of *Pseudomonas fluorescens* ATCC 27663 (a), *Pseudomonas facilis* ATCC 17695 (b), *Bacillus cereus* ATCC 14893 (c), *Sphingomonas capsulate* ATCC 14666 (d), *Aquaspirillum dispar* ATCC 27510(e), *Sinorhizobium meliloti* DSM 1981(f), *Arthrobacter globiformis* ATCC 8010 (g) and *Thermus filiformis* Neu 1128 (h). GELCOMPARE II software (Saint-Martens-Latem, Belgium) version 4.0. was employed for the analysis of the DGGE profiles.

Results and discussion

Bio-inoculants (BI) were identified by band excision and sequencing. This allowed to compare if bands with the same mobility were also present in the rhizosphere profiles (Figure 4). Bands with similar mobility than the bio-inoculant *Pseudomonas putida* YP2 were observed in all the profiles, however this band was expected to be found only in roots inoculated with *P. putida* YP2 (lane III in soil and in compost). One of these bands was excised (band indicated as 1) and later identified as *Herbidospora sakaeratensis* (100% identity). A similar observation was made for the bio-inoculant *Rhizobium pusense* YP3 that displayed two bands (lane BI), from which only one with a similar mobility was detected in almost all the roots in soil and in compost. One of these bands (indicated as 2) was identified with equal certainty as *Pantoea vagans* and *Escherichia hermannii* (100% of identity), confirming that it was not related to the bio-inoculant *R. pusense* YP3. Although V3-DGGE profiles did not allow to verify root colonization by bio-inoculants, this analysis offered an interesting insight into the bacterial populations associated to the roots of the seedlings. Since similar profiles were observed among the roots in soil and in compost, the most intensive bands (present in all profiles) were excised and sequenced. Band 3 could be assigned with a low identity (91%) to the cyanobacterium *Chroococcidiopsis thermalis*, band 4 to *Mycobacterium smegmatis* (99%), band 5 to *Methylobacterium extorquens* (97%) while band 6 to *Novoshingobium* sp. (100%).

According to these results, we conclude that higher sensible techniques such as quantitative PCR rather than fingerprinting approaches should be performed to trace and detect the prevalence of these inoculants strains in soils.

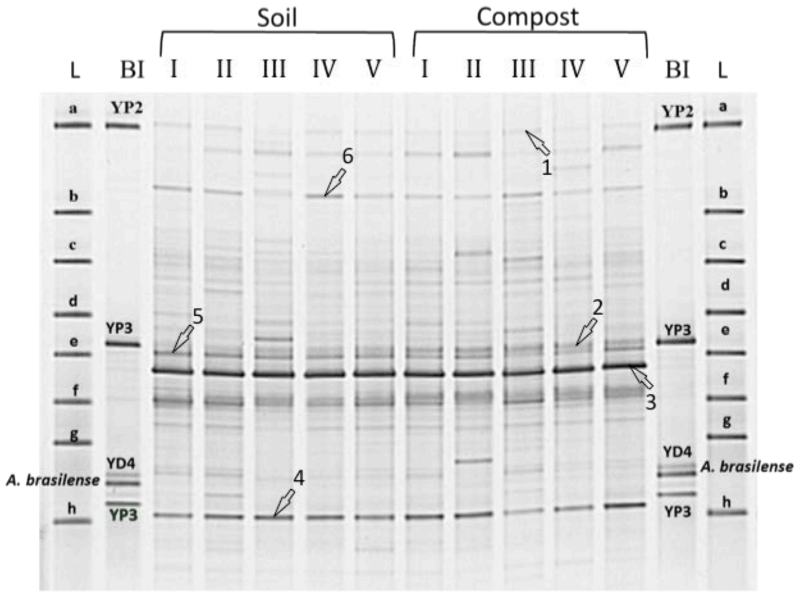

Figure 4. DGGE analysis of the V3 region 16S rRNA gene of Yerba Mate roots bio-inoculated in soil and compost after 2 months of cultivation. Bio-inoculants (BI) were identified by band excision and sequencing: *Pseudomonas putida* (YP2), *Rhizobium pusense* (YP3), *Kosakonia radicincitans* (YD4) and *Azospirillum brasilense*. Lanes I-V of soil and compost correspond to roots inoculated with: (I) *A. brasilense* 245, (II) YD4, (III) YP2, (IV) YP3 and (V) C-.Common bands in the profiles were identified as: *Herbidospora sakaeratensis* (1), *Pantoea vagans* and *Escherichia hermannii* (2), *Chroococcidiopsis thermalis* (3), *Mycobacterium smegmatis* (4), *Methylobacterium extorquens* (5) and *Novoshingobium sp* (6).

2.8.4. Mycorrhizae associations in Yerba Mate

Yerba Mate presents associations with arbuscular-vesicular mycorrhizae (VAM) fungi (Gaiad and Sidney Lopes 1986, Andrade et al. 2000). In a nursery experiment, a colonization rate of 70% of young roots has been observed, in which the genera *Acaulospora* and *Glomus* were the most frequent (Gaiad and Sidney Lopes 1986). It is well-reported that bio-inoculation with a consortia of PGPR and mycorrhizae have a marked effect on plant growth (Chanway and Holl 1991, Sing and Adholeya 2003, Mäder et al. 2011). The promising results of the bio-inoculation assay in nursery open up the possibility of a synergic PGPR-mycorrhizae effect on plant growth as discussed in the manuscript presented in Chapter 2. Therefore, we decided to analyze if the roots of seedlings of the bio-inoculation assays were colonized by mycorrhizae to take into consideration a co-inoculation strategy for a future plant bio-inoculation assays.

Methods

When the bio-inoculation assay in nursery was harvested, roots of seedlings were stained to analyze mycorrhizal colonization according to the protocol of Vierheilig and Piché (1998).

Results and discussion

Colonized mycorrhizae roots were observed in seedlings from the bio-inoculation assay in nursery (Figure 5). Despite the fact that the root colonization rate of each bio-inoculation treatment was not determined, neither the presence nor identification of mycorrhizal spores in soil and in compost, we decided to analyze these traits in a new bio-inoculation assay. Dr Alok Adholeya, an expert in mycorrhizal inoculants, suggested to analyze the co-inoculation effect of mycorrhizae and our native PGPR strains on the growth of Yerba Mate seedlings in nursery. They provided us with a mixed consortia of *Acaulospora scrobiculata, Acaulospora leavis, Glomus intraradices* and *Gigaspora margarita.* A mixed bio-formulate of this mycorrhizal consortia and *Kosakonia radicincitans* YD4 strain was inoculated in Yerba Mate seedlings in nursery (August 2013, Santo Pipo, Argentina). However, after one month of cultivation the seedlings did not survive. We hypothesized that the seedlings were too young (2 months-old) at the moment of inoculation.

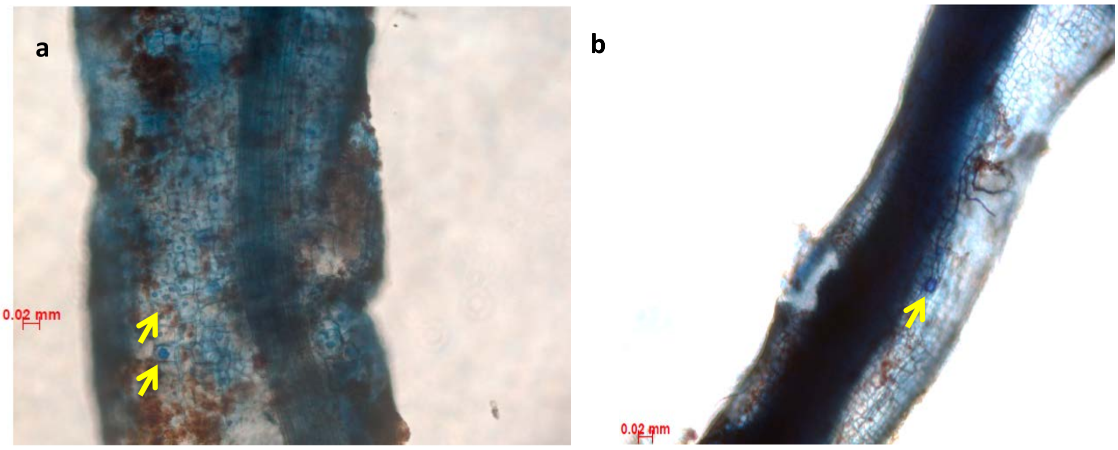

Figure 5. Mycorrhizae associations in Yerba Mate roots. Arrows indicate the presence of vesicles inside root cells in (a) and germinated spores in (b).

2.8.5. Appendix references

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Chapter 3: Potential rhizosphere colonization ability and safety of the PGPR strain YD4

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Genome Sequence of *Kosakonia radicincitans* Strain YD4, a Plant Growth-Promoting Rhizobacterium Isolated from Yerba Mate (*Ilex paraguariensis* St. Hill.)

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***Kosakonia radicincitans* strain YD4 is a rhizospheric isolate from yerba mate (*Ilex paraguariensis* St. Hill.) with plant growth-promoting effects on this crop. Genes involved in different plant growth-promoting activities are present in this genome, suggesting its potential as a bioinoculant for yerba mate.**

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Yerba mate is an important southern South American crop, used to produce an energizing beverage, widely consumed in this region as an alternative to coffee. Due to mate tea's high antioxidant content and nutritional benefits for human health, its popularity is rapidly increasing, with expansion to new markets, including Europe, Asia, and the United States (1). Argentina is the leading producer of yerba mate with plantations mainly clustered around the Northeast of the country. Currently, producers are struggling with soil degradation due to inadequate agricultural practices. Bioinoculation of yerba mate with native bacterial isolates was recently demonstrated as a sustainable agricultural practice to improve plant growth (V. Bergottini, M. Otegui, D. Sosa, P. Zapata, M. Mulot, M. Rebord, J. Zopfi, F. Wiss, B. Benrey, and P. Junier, submitted for publication).

The sequenced bacterium was isolated from the rhizosphere of yerba mate in a soil-degraded plantation in Argentina. The strain YD4 was selected on the basis of its plant growth-promoting effect on yerba mate seedlings growing in a nursery, reflecting its potential as a bioinoculant (Bergottini et al., submitted). The 16S rRNA, *atpD* and *rpoB* gene sequences of YD4 showed the highest homology with the plant growth-promoting endophyte *Kosakonia radicincitans* DMS16656^T (2). The sequencing and annotation of the genome aimed at a broader understanding of the plant-growth promotion mechanisms and the safety verification for using this strain as a bioinoculant.

Genomic DNA was extracted from an overnight culture using the Genomic-tip 20/G kit (Qiagen GmbH, Germany). Sequencing was performed with the PacBio RS II system based on single molecule, real-time (SMRT) technology (Pacific Biosciences, California). The draft genome of *K. radicincitans* YD4 presents a unique contig of 5,226,863 bases, and a G+C content of 54.3%. Genome annotation was performed using an Ergatis-based (3) workflow with minor manual curation and visualized with Artemis Genome Browser and Annotation Tool (4). A total of 4,836 coding sequences (CDSs), 82 tRNAs, and 7 rRNAs were predicted.

The plant growth-promoting abilities of the strain YD4 experimentally observed were consistent with the presence of genes for nitrogen fixation (*nifQBALFMZWVSUXNEYTKDHJ*), for the mineralization of organic phosphates (*phoC*), siderophore production (*entABCDEF*) and uptake (*fepABCDG* and *fhuFCDBE*), an indolpyruvate decarboxylase (*ipdC*) involved in the synthesis of the auxin indol acetic acid (IAA), and an auxin efflux carrier. Most of these genes were also described in the *K. radicincitans* DMS16656^T genome (2). Similar to the endophytic nitrogen-fixing strain *Enterobacter* sp. SP1 (5), *K. radicincitans* YD4 presents genes for the type III secretion system, suggesting that a broader analysis of these genes will be necessary in order to infer a potential mutualistic or parasitic lifestyle in this strain.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. JSFC00000000. The version described in this paper is version JSFC01000000.

ACKNOWLEDGMENTS

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3.1. Summary

For biotechnological applications a PGPR strain should be able to persist in the rhizosphere and should not represent a potential sanitary risk for the environment and human health. In this study, the whole genome of the PGPR strain YD4 was sequenced and annotated. This allowed to analyze the genetic potential to be a rhizosphere competent strain, as well as the presence of virulence associated genes that could suggest a potential pathogenic lifestyle. The potential ability to colonize successfully the rhizosphere was demonstrated by the presence of genes involved in chemotaxis, motility, adherence structures and catabolism of plant-derived compounds. Regarding the safety of using YD4 as bio-inoculant, genes for the Type III (T3SS) and Type VI (T6SS) secretion systems, often associated with virulence in some bacterial species, were identified. We hypothesize that both secretion systems are not a pre-requisite for virulence given their distribution in the *Kosakonia radicincitans* strains analyzed, since the phytopathogenic strain *K. radicincitans* UMEnt01/12 does not present T3SS, whereas the plant growth promoting endophyte *K. radicincitans* DSM 16656^T presents T6SS. Since the role of both secretion systems has been associated with an adaptation and survival to a particular bacterial niche in previous studies (Tampakaki 2014, Schwarz et al. 2010), we hypothesize that they might contribute to the competitiveness against other microbes and protists in the rhizosphere. Combining these analyses, we hypothesize that *K. radicincitans* YD4 present the genetic potential to colonize and survive competitively the rhizosphere niche.

3.2. Introduction

The genomes of several PGPR strains have been sequenced and analyzed (Gan et al. 2012, Liu et al. 2012, Zhu et al. 2012, Taghavi et al. 2010, Gupta et al. 2014) reflecting the need to determine the genetic background of plant-bacteria interactions responsible for improving the growth and health of many crops worldwide. All the genomic information available is a very valuable source for comparative analysis, not only to confirm the presence or absence of specific genes or genomic regions within a species, but also to infer evolutionary hypothesis about a mutualistic or pathogenic bacterial lifestyle in relation to its plant host. There are four available whole genome projects of *Kosakonia* deposited in the NCBI, from which three of them belong to plant growth promoting bacteria, including *Kosakonia radicincitans* DSM 16656^T isolated from winter wheat (Witzel et al. 2012), *Kosakonia sacchari* SP1 isolated from sugarcane (Zhu et al. 2012) and *Kosakonia oryzae* strain:KO348 isolated from rice (BioSample SAMN03354161 in NCBI). In contrast, *Kosakonia radicincitans* UMEnt01/12 is a plant pathogen isolated from banana (Mohd Suhaimi et al. 2014).

It has been demonstrated that the plant growth promoting rhizobacteria (PGPR) YD4 can increase the biomass yields of Yerba Mate seedlings in nursery by as much as 183% (Chapter 2). Besides the plant growth and/or biocontrol traits, a bacterial inoculant should be able to colonize the rhizosphere and should not represent a sanitary risk for humans and the environment. Since YD4 bio-inoculation resulted in plant growth promotion in Yerba Mate seedlings, we hypothesize that this strain presents potential rhizosphere competence traits that could be inferred from its genome. In addition, considering the fact that a close relative strain *K. radicincitans* UMEnt01/12 is a phytopathogen in another plant species, it is crucial to evaluate if YD4 could present virulence-associated genes. Therefore, the aim of this study was to analyze in the genome of YD4 the presence of genetic elements necessary to infer: i) a potential rhizosphere colonizing ability and, ii) a non-pathogenic lifestyle in this bacterial inoculant. Moreover, the presence of genes associated to plant growth promoting traits was analyzed.

3.3. Material and methods

Phylogeny

We reconstructed the phylogeny of strain YD4 by analyzing the partial sequences of the 16S rRNA and the housekeeping genes *atpD* and *rpoB* of closely related species used in a previous study (Brady et al. 2013). Sequences alignment was performed with MAFFT software version 7.0 (Katoh and Standley 2013). The aligned sequences were analyzed with PhyML software, version 3.0 (Guindon et al. 2010) using LG (proteins) and GTR (16S rRNA gene) substitution models with maximum-likelihood estimation for all parameters, twelve types of mutation rates and support values aLRT. Then, trees were obtained and processed with Newick Utilities (Junier and Zdobnov 2010). In addition, the core genome of *Kosakonia* and *Enterobacter* strains available in the web interface of the Efficient Database framework for comparative Genome Analyses using Blast score Ratios (EDGAR) was used to obtain a phylogenetic tree according to Blom et al. 2009. Multiple alignments for all core genes were created with MUSCLE (Edgar 2004). The matching genes were concatenated to one big multiple alignment and finally a phylogenetic tree is generated with PHYLIP (Felsenstein 1989). Tree rendering was performed with the ScripTree web interface (Chevenet et al. 2010).

Comparative analysis

The plant growth promoting (PGP) traits observed in the *in vitro* tests, rhizosphere competence traits and virulence-associated genes were analyzed in YD4 genome by comparative genomics. We included in the analysis the available whole genome projects of: two *K. radicincitans* strains DSM 16656^T (Witzel et al. 2012) and UMEnt01/12 (Mohd Suhaimi et al. 2014); plant growth promoting strains of the genus *Enterobacter* (closest relative of *Kosakonia*): *Enterobacter* sp. 638 (Taghavi et al. 2010), *Enterobacter cloacae* subsp. *cloacae* ENHKU01 (Liu et al. 2012), the phytopathogenic strain *Enterobacter cloacae* EcWSU1 (Humann et al. 2011) and the human pathogen *Enterobacter cloacae* subsp. *cloacae* ATCC13047 (Ren et al. 2010). The core genome, pan genome, singletons analysis and Venn diagram construction were obtained with EDGAR (Blom et al. 2009) using *K. radicincitans* strain YD4 as reference strain.

Metabolization of carbon sources

The ability of the bacterial strain YD4 to utilize different carbon sources was analyzed by API20 NE test (Biomérieux).

3.4. Results and discussion

Phylogenetic analysis

Previous to perform the comparative analysis, the phylogenetic affiliation of strain YD4 was inferred. In the 16S rRNA and the concatenated *atpD* and *rpoB* genes phylogeny, YD4 clustered with other *K. radicincitans* strains (Figure 1). This phylogenetic clustering is in good accordance with the phylogenomics analysis, in which two bigger clusters can be distinguished between *Enterobacter cloacae* and *K. radicincitans* genomes (Figure 2). These results confirm the fact of the need to transfer *Enterobacter radicincitans* from *Enterobacter* to the new reassigned genus *Kosakonia* (Brady et al. 2013). To obtain more information about the relationship of strain YD4 with the other *K. radicincitans* strains, the synteny between all the genome pairs was plotted using the Edgar web platform (Blom et al. 2009). This allowed to obtain an overview on the gene conservation order between individual strains (Blom et al. 2009) (Figure 3). The comparisons have shown more conservation order between the plant growth promoting strains YD4 and DSM 16656^T, even though some large-scale inversion events occurred. In contrast, the number of rearrangements increased between the genomes of DSM 16656^T and UMEnt01/12 strain that are closer relatives.

General features of K. radicincitans genomes

The general features of the genomes are summarized in Table 1. Strains YD4 and UMEnt01/12 present both one unique contig of 5.23 Mbp and 5.78 Mbp, respectively; whereas strain DSM 16566^T possesses two contigs with a total size of 6 Mbp. The GC content among strains ranged from 53-54.3% and the predicted coding sequences (CDS) are similar between strain YD4 and UMEnt01/12 (4.836 and 4.957 CDS respectively), but higher for strain DSM 16566^T (6.124 CDS).

The pan genome of *K. radicincitans* is represented in a Venn diagram (Figure 4) and consists in 6467 CDSs, from which 4233 CDSs conform the core genome representing approximately the 65.46% of all CDS. The percentage of unique genes shared between strain YD4 and DSM 16656^T (1.44%) is similar to that shared between strain YD4 with UMEnt01/12 (1.33%). However, a larger number of genes are shared between DSM 16656^T and UMEnt01/12 representing the 8.9% of the pan genome not present in strain YD4. Among the singletons of YD4 we found a gene cluster encoding for the Type III secretions system (T3SS), a putative prophage gene cluster and several hypothetical proteins.

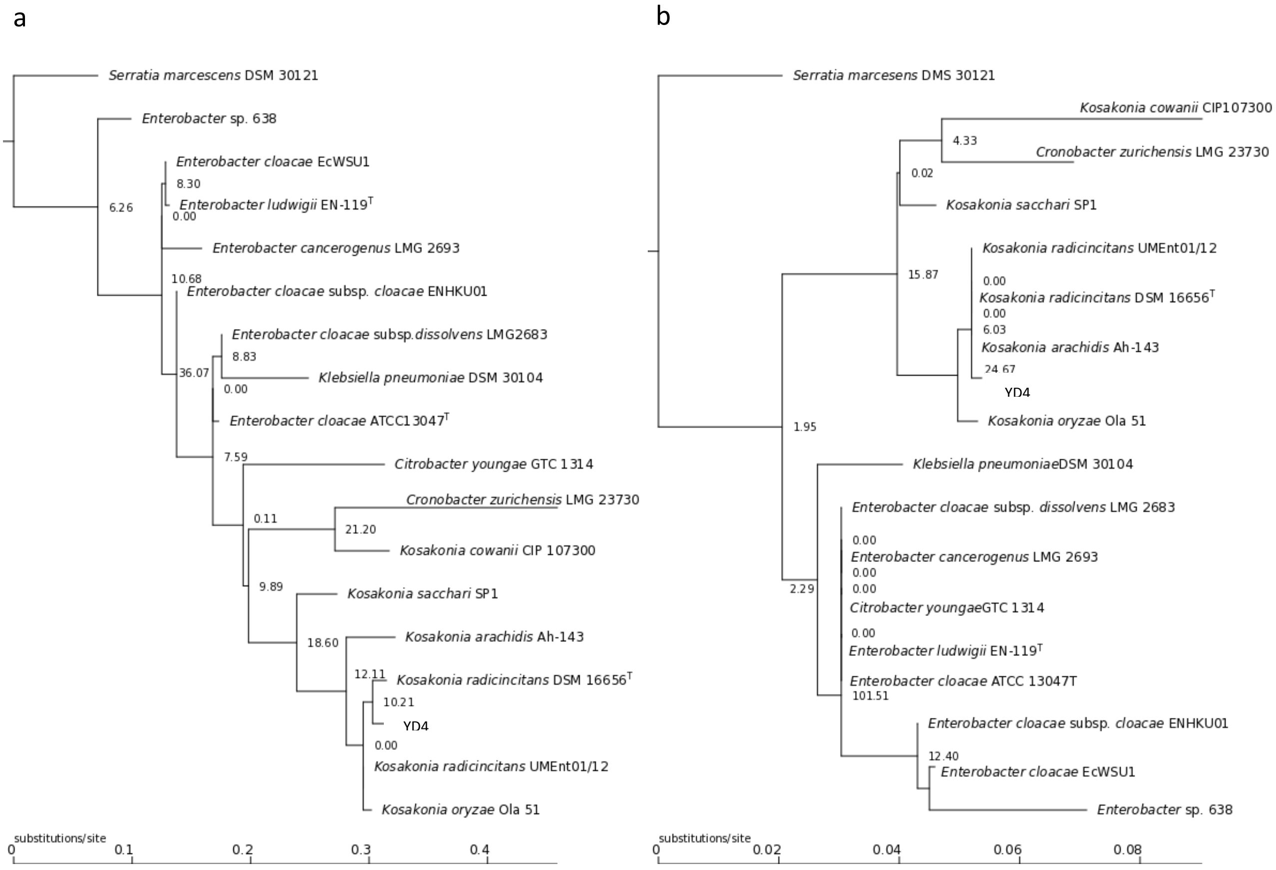


Figure 1. Maximum likelihood trees based on partial 16S rRNA gene sequences (a) and on concatenated partial *rpoB* and *atpD* genes sequences (b) of *Enterobacter and Kosakonia* species.

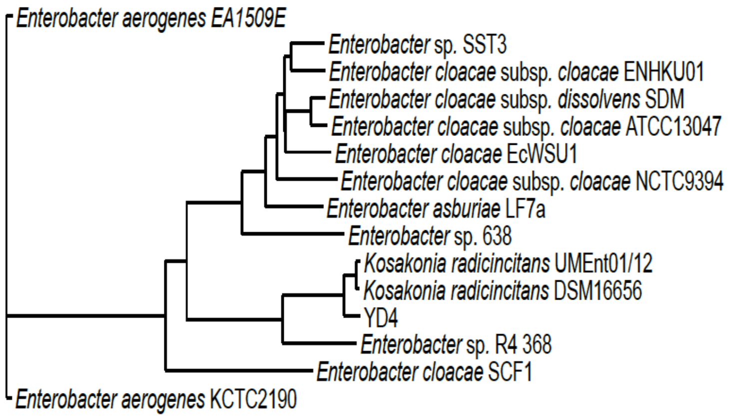
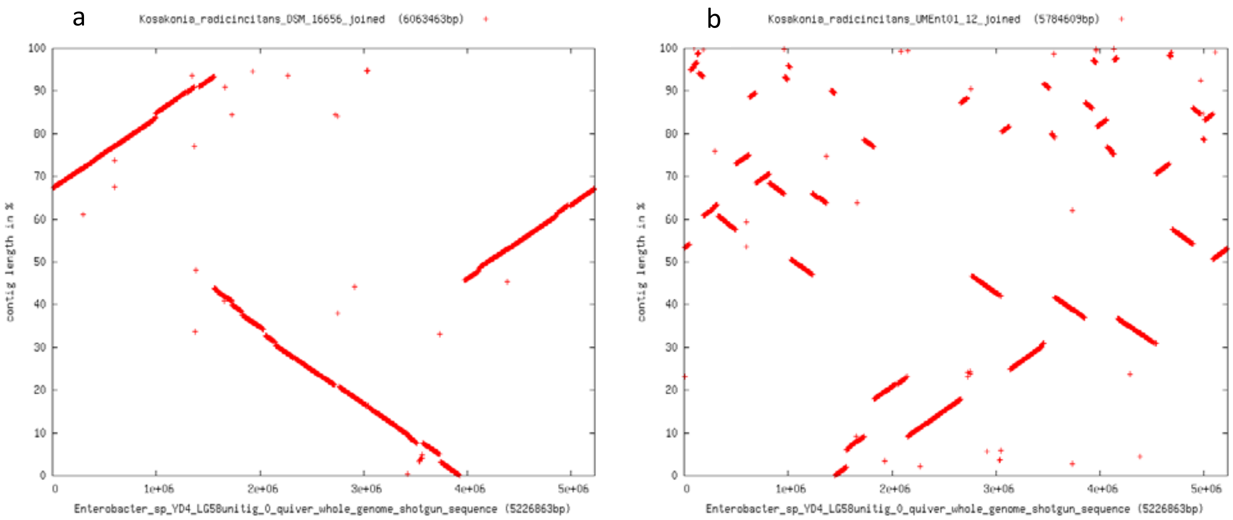


Figure 2. Phylogenetic tree of *Kosakonia* and *Enterobacter* genomes available in the web interface of EDGAR. Based on the core genome of 1947 CDS the divergence of strain YD4 was estimated.

Table 1. General features of the three genomes of *Kosakonia radicincitans.*

General features	YD4	DSM 16656*	UMEnt01/12*
Genome size (bp)	5.23 Mbp (1 contig)	6 Mbp (2 contigs)	5.78 Mbp (1 contig)
GC content	54.3%	53%	53.9%
CDSs predicted	4836	6124	5463
tRNAs	82	69	75
rRNAs	7	9	9

* The genome's features of DSM 16656 were obtained from Witzel et al. 2012, and of UMEnt01/12 from Mohd Suhaimi et al. 2014.



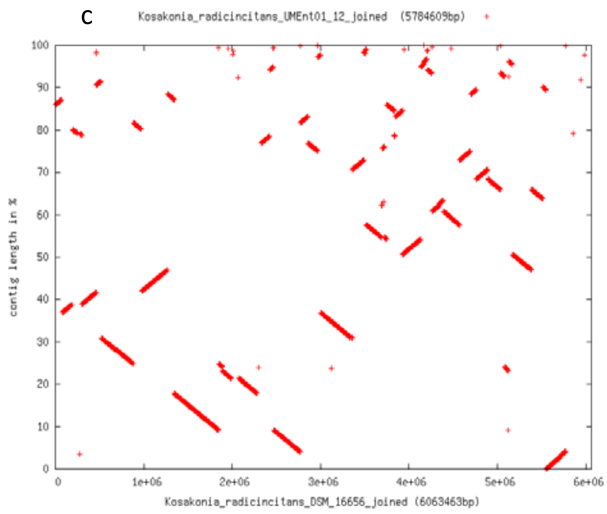


Figure 3. Synteny of *Kosakonia radicincitans* strains using EDGAR. The position of each CDS of the genome given on the X axis is plotted against the position of its homologue in the second genome given on the Y axis (Blom et al. 2009). A diagonal plot indicates identical genomes. The names of the analysed genomes pairs are given on top of each plot. (a) Between YD4 and DSM 16656^T there are chromosomal rearrangements indicating large-scale inversions events. An important increase in the number of rearrangements can be observed between (b) YD4 - UMEnt01/12 and (c) DSM 16656^T - UMEnt01/12, in which gene order seems less conserved.

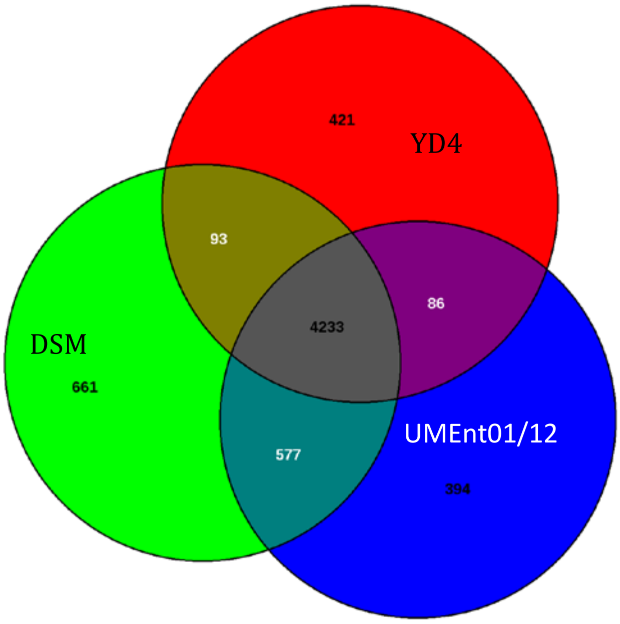


Figure 4. Venn diagram representing the pan genome of *K. radicincitans* strains YD4, DSM16656^T, and UMEnt01/12 using EDGAR (Blom et al. 2009). Overlapped regions represent the CDS numbers shared among *K. radicincitans* genomes and not overlapped regions the CDS without reciprocal best hit to the other genomes.

Rhizosphere competence traits

Besides the plant growth promoting traits, colonization and survival within the rhizosphere are important traits in an effective bio-inoculant. Rhizosphere competence traits can be grouped basically in three categories: chemotaxis/motility, adherence and growth. For motility, we confirmed the presence of two flagellar biosynthesis operons and chemostaxis genes (*flgNMABCDEFGHIJKL, che, fhDC, motAB, cheAW, fliYZA, fliDST, fheEAB, cheZBR, fliEFGHIJKLMNOPQR, fhlCD;* and *fliRQPONMLKJIHGFE fliTSD fhlDC motAB cheAW cheBYZ flhBAE flgLKJIHGFEDCBAMN)* in YD4 genome with a similar gene organization than in other enteric bacteria (Liu and Ochman 2007). Putative genes involved in twitching motility were also identified. For adhesion mechanisms, seven gene clusters involved in pili/fimbria biosynthesis were identified. Within some of these clusters, many hypothetical proteins are present as well as some antibiotic resistance genes (beta-lactamase) and chemotaxis genes. The ability to metabolize plant-derived compounds is also a rhizosphere competence trait. We identify genes that can be associated with the transport and metabolization of the following carbon sources: glucose, fructose, mannose, galactose, rhamnose, arabinose, xylose, maltose and lactose, suggesting that YD4 can use a variety of plant-derived compounds. This genetic potential was consistent with the results obtained with an API test analysis (Biomerieux) in which YD4 assimilated D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose and sucrose. According to these results, we hypothesize that strain YD4 presents the potential to be rhizosphere competent and therefore, might successfully colonize the rhizosphere.

Virulence genes

Comparative analysis of virulence genes in many pathogenic bacteria, including human and plant pathogens, allowed to infer if a potential pathogenic lifestyle could be developed in YD4. In some bacterial species the presence of the secretions systems Type III (T3SS) and Type VI (T6SS) is associated with their pathogenicity (Tampakaki 2014, Shrivastava and Mande 2008). These three secretion systems are implicated in interactions between bacterial and eukaryotic cells in which bacterial effectors are released directly into the eukaryotic cytosol (Gerlach and Hensel 2007).

A gene cluster encoding for a T3SS was identified in YD4, but not in the other two *K. radicincitans* strains (DSM 16656^T and UMEnt01/12). T3SS is commonly encoded in a large cluster of contiguous genes, which are often considered pathogenic islands (i.e. T3SS pathogenic island in *Salmonella typhimurium*) (Kimbrough and Miller 2000). According to our results T3SS cannot be always associated with pathogenicity since it is absent in two closest phytopathogens strains, *K. radicincitans* UMEnt01/12 (causing wilt disease in banana) and *E. cloacae* EcWSU1 (causing bulb decay in onion). Despite the long tradition to associate T3SS to virulence mechanisms, it has been demonstrated that T3SS also plays a key role in mutualistic symbiosis and commensalism (Dale and Moran 2006, Preston 2007). Therefore, its function in YD4 genome remains unclear.

Two gene clusters for T6SS are present in YD4 and in *K. radicincitans* strains, DSM 16656^T and UMEnt01/12. These two clusters exhibit a particular genetic organization commonly found in other *Enterobacter cloacae* species (Liu et al. 2013). T6SS is expected to enhance the survival of non-pathogenic bacterial strains in their habitats by providing defense against other simple eukaryotic cells and other bacteria (Schwarz et al. 2010). T6SS genes may be found in many soil bacteria that are not necessarily pathogens or symbionts, i.e. *Pseudomonas putida* and *Myxococcus xanthus* (Bingle et al. 2008). We hypothesize that these two T6SS clusters conserved within *K. radicincitans* and *Enterobacter cloacae* strains represent a fitness advantage to survive and colonize successfully many different types of ecological niches.

Plant growth promotion traits

The presence of genes involved in plant growth-promotion (PGP) was analyzed in the genome of YD4 in comparison with other plant growth promoting bacteria (Table 1). N_2 fixation can be expected as one of the mechanisms of plant growth promotion of YD4. We identified in the genome of YD4 the whole operon for N_2 fixation (*nifQBALFMZWVSUXNEYTKDHJ*) similarly to *K. radicincitans* DSM 16566T and UMEnt01/12 strains. However this operon is absent in all of the *Enterobacter* sp. strains compared. Moreover, genes for the dissimilation of nitrate (*narLXKGHJI*), nitrite (*nirBD*), nitrate/nitrite and ammonium transport were identified in all genomes.

Among the mechanisms of phosphate solubilisation described so far, the molecular basis of phosphate solubilisation via gluconic acid production is well known (Goldstein 1995). We analyzed the presence of genes involved in this mechanism and found a membrane glucose dehydrogenase-encoding gene, however the whole operon for the biosynthesis of the cofactor pyrroloquinoline-quinone (PQQ) was not founded. All *K. radicincitans* strains present a putative PQQ biosynthesis protein, thus further deeper analysis should be performed to identify the entire operon. On the other hand, a gene involved in organic phosphate mineralization (*phoC*) was identified in all *K. radicincitans* genomes. A considerable phosphatase activity has been observed in the rhizosphere of different crops in acidic and neutral soils (Rodriguez and Fraga 1999). This activity might contribute to facilitate phosphorus uptake by the plant host.

Siderophore production is another key trait in many rhizobacteria due to its involvement in facilitating iron uptake by plants and limiting the iron availability to other competitors in the rhizosphere (Kloepper et al. 1980). Similarly than in *Enterobacter* sp. 638 (Liu et al. 2013), YD4 genome presents a gene cluster for the biosynthesis of the siderophore enterobactin (*entABCDEF*), its secretion exporter (*entS*), the uptake system of the iron-enterobactin complex (*fepABCDG, exbBD*) and a hypothetical enterobactin esterease (*fes*) necessary to release iron from the enterobactin-iron complex. This siderophore gene cluster was conserved in *K. radicincitans* strains, whereas the aerobactin cluster is only present in *Enterobacter* strains. Moreover, a gene cluster for ferrichrome uptake (*fhuFCDBE*) was found in *K. radicincitans* genomes.

The ability to synthesize the phytohormone auxin indole acetic acid (IAA) is another common trait in rhizobacteria. The main precursor for IAA synthesis is tryptophan and five different biosynthetic pathways have been described so far (reviewed in Spaepen and Vanderleyden 2011). In YD4 genome, genes involved in the indole-3-pyruvate pathway, the indolpyruvate decarboxilase (*ipdC*) and an indole-3-acet-aldehyde dehydrogenase, as well as a putative auxin efflux carrier are present. These genes were also present in all the other genomes analyzed.

Although YD4 did not exhibit an antagonist activity *in vitro* against species causing damping-off, we hypothesize that potential antimicrobial activities could be present in its genome since this is a common trait observed in many *Enterobacter* sp. plant associated bacteria (Liu et al. 2013, Taghavi et al. 2010). Genes involved in bacteriocin biosynthesis Colicin V and bacteriocin production cluster (Riley and Wertz 2002) as well as tolerance genes to Enteridicin A and B (Bishop et al 1998) were identified in YD4 similarly to those in other *Enterobacter cloacae* strains (Liu et al. 2013). Additional genes associated with bacteriocin production (*rhsB*) are also present in the variable region of T6SS gene cluster as observed in other *E. cloacae* strains (Liu et al. 2013). Moreover, in the same clusters genes involved in cell wall degradation *LysM* (peptidoglycan-binding proteins) and antifungal activity (chitinases) were identified (Liu et al. 2013).

3.5. Conclusion

The whole genome analysis of the PGPR strain YD4 revealed that this strain presents several potential plant growth promoting mechanisms, including a marked antimicrobial antagonism potential that was not revealed in the *in vitro* PGPR tests. In addition to this, many functions related to the potential capability to survive and colonize successfully the rhizosphere have been identified. The presence of genes involved in motility/chemotaxis and adherence to surfaces reflects the potential rhizosphere competence of strain YD4. Moreover, the possession of T3SS and T6SS suggests potential adaptive mechanisms in YD4 to its ecological niche, the rhizosphere. Despite the fact that these genes are the key virulence factors in many pathogenic bacteria, they have been reported in many soil non-pathogenic bacteria.

3.6. Acknowledgments

I would like to thank Thomas Junier for his collaboration with the phylogeny analysis.

Table 2. Comparison of plant growth-promoting traits and rhizosphere competence traits among *K. radicincitans* and *Enterobacter* strains. Table adapted from Liu et al. (2013) in which *K. radicincitans* strains YD4, DSM 16656^T, and UMEnt01/12 were included for this study.

			***Kosakonia* strains**		***Enterobacter* strains**				
Traits and genes	**Pan genome position**	**Locus in YD4**	**YD4**	**DMS16656^T**	**UMEnt01/12**	**ENHKU01**	**638**	**EcWSU1**	**ATCC13047**
N$_2$ fixation, *nif*	1436713-1454925	LG58_1511- 1529	+	+	+	-	-	-	-
Nitrate/nitrite dissimilation *nar* and *nir*	1280107-1289967	LG58_1350-1355	+	+	+	+	+	+	+
P solublization, *pqq* operon			-	-	-	-	-	-	-
P mineralization *phoC*	1668004	LG58_1775	+	+	+	-	-	-	-
IAA *ipdC/* efflux carrier	494419	LG58_551	+	+	+	+	+	+	+
Siderophores *ent, fep, exb,fes*	2305447-2321538	LG58_2448-2459	+	+	+	+	+	+	+
Flagella (*flg, fli*)	169393-184525	LG58_197-216	+	+	+	-	-	-	+
Chemotaxis (*che*)	1770139-1781142	LG58_1890-1903	+	+	+	+	+	+	+
Type III	4397305-4413462	LG58_4716-4734	+	-	-	-	-	-	-
Type VI	504094-531264	LG58_559-582	+	+	+	+	+	-	+
	1052935-1076304	LG58_1139-1159	+	+	+	+	-	+	+
Tolerance to Entericidin A/B	3296662	LG58_3512	+	+	+	+	+	+	+
Colicin V and bacteriocin production cluster	567511	LG58_626	+	+	+	+	+	+	+
	3873895	LG58 4139	+	+	+	-	-	-	-
	4493725	LG58_4824	+	+	+	-	-	-	-
Chitinases	516193	LG58_568	+	-	-	-	-	-	-
	2569294	LG58_2747	+	+	+	-	-	-	-
	5662378		-	-	-	+	-	+	+
Bacterial wall degradation (*LysM)*		LG58_1058	+	+	+	+	+	+	+
		LG58_1857	+	+	+	+	+	+	+
		LG58_3456	+	+	+	+	+	+	+
		LG58_3538	+	-	-	-	+	-	-
		LG58_4821	+	+	+	+	+	+	+

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Chapter 4: Monitoring the colonization and persistence of the PGPR strain YD4 in the rhizosphere

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4.1. Summary

Aside of exerting a plant growth-promoting effect a bacterial inoculant should be rhizosphere competent, in other words, should be able to compete successfully against the native microflora to colonize and establish in the rhizosphere. For biotechnological applications it is relevant to evaluate the dynamics of an introduced bacterial strain since its ability to persist in the soil is a key factor for selecting a bio-inoculant. In this study, we monitored the dynamics of the introduced bacterial strain *K. radicincitans* YD4 in the rhizosphere of Yerba Mate seedlings in nursery during 5 weeks by performing two different real time PCR approaches. One of them, in which a novel strain-specific primer set was designed, allowed to detect and quantify the strain YD4 in the rhizosphere. According to our results, the introduced strain was able to colonize and persist within the rhizosphere during the 5 weeks that the experiment lasted. After its application in soil (5 ml of a bacterial suspension of 10^8 cells mL^{-1} in pots of 200 cm^3), YD4 population remained almost constant in the rhizosphere and reached 5.77 x 10^4 copy numbers per gram of root at the end of the experiment (fifth week). This study allowed to attribute the plant growth-promoting effect observed in seedlings with the presence of the introduced bacterial inoculant YD4 within the rhizosphere. In addition, this study provides a strain-specific qPCR approach to monitor this bacterial inoculant in soil.

4.2. Introduction

Effective bacterial inoculants are those that aside promoting plant growth or protection against phytopathogens are able to survive and colonize successfully the rhizosphere. Many bacterial strains with promising plant growth promotion effects in growth-chamber assays have failed in greenhouse and field experiments due to their ineffectiveness in colonizing the rhizosphere and roots (Benizri et al. 2001, Bloemberg and Lugtenberg 2001). This phenomenon can be explained by a lack of adaptation to local edaphic and environmental conditions, and therefore a decrease in competitiveness with respect to the native bacterial community for the establishment and persistence in soil. Hence, evaluating the ability of an introduced bacterial inoculant to colonize and persist in rhizospheric soil is relevant to establish their potential as an effective plant growth promoting rhizobacteria (PGPR).

Kosakonia radicincitans YD4 is a PGPR strain that has shown to increase biomass yields of *Ilex paraguariensis* St. Hill. (Yerba Mate) seedlings in nursery (Bergottini et al. 2015), representing a candidate bio-inoculant for future biotechnological applications. The genome analysis of *K. radicincitans* YD4 revealed a potential ability to colonize the rhizosphere since many genes involved in chemotaxis, motility, attachment, antimicrobial activity and siderophore production are present in this strain. We hypothesize that the growth promotion effect observed on Yerba Mate seedlings is caused by the introduced inoculant *K. radicincitans* YD4 upon the successful colonization of the rhizosphere of this crop. Therefore, the aim of this study was to detect and quantify, using a strain-specific quantitative real time

PCR approach, the introduced PGPR *K. radicincitans* YD4 in the rhizosphere of Yerba Mate seedlings.

4.3. Material and methods

Bio-inoculation assay

A plant bio-inoculation assay was performed in a nursery (Santo Pipó, Misiones, Argentina) from November 2014 to January 2015 (summer season) using five-month old Yerba Mate seedlings grown in pots (200 cm^3) with approximately 150 g of soil (Ultisol). The soil presented a pH of 6.52, 28700 mg kg^{-1} of C, 2700 mg kg^{-1} of N, 13.81 of C/N, 34.43 mg kg^{-1} of P, 203.3 mg kg^{-1} of K, 3134 mg kg^{-1} of Ca, 414.4 mg kg^{-1} of Mg, and 23 mg kg^{-1} of Na. Inoculation was performed by irrigating the soil near the roots with 5 ml of a fresh bacterial suspension (in water) adjusted to an abundance of 10^8 cells mL^{-1}. Control plants were irrigated with water. Bio-inoculation and control treatments were analyzed on 50 seedlings each. Two months post inoculation, seedlings were harvested to determine shoot and root dry weight, height, and the concentration of macronutrients in shoots. To determine the content of macronutrients in shoots, plants were sorted in 5 replicates (n=10) per treatment to measure N by the semi-micro-Kjeldahl method (Kjeldahl 1833); P by the vanadate/molybdate method (Chapman and Pratt 1962); K by flame photometry; and Ca and Mg by EDTA titration (Bock 1962). The statistical significance of the effect of bio-inoculants on plant biomass and in the macronutrients content in shoots was analyzed by Wilcoxon tests.

DNA extraction

From the day of inoculation and every 7 days during 5 weeks, three seedlings per treatment were harvested randomly to quantify the cell numbers of strain YD4 in the rhizosphere of seedlings. Roots were shaken vigorously to remove non-rhizospheric soil, and three grams of roots per seedlings were used to perform DNA extraction with the FastDNA Spin Kit for Soil (MP Biomedicals, California), according to a modified protocol (Wunderlin et al. 2013). DNA concentration was measured with a Qubit Fluorometer using a dsDNA HS Assay Kit (Invitrogen, California). The concentration of all samples was adjusted by dilution to 2ng/µl.

Quantitative PCR assays

In order to calculate the ratio of the YD4 inoculum to the total bacterial load of each sample, two quantitative PCR (qPCR) assays were used. First, for quantifying total bacteria, qPCR amplification of the V3 hyper-variable region from the 16S rRNA gene was carried out using the primers 338f (5’-ACTCCTACGGGAGGCAGCAG-3’) and 520r (5’-ATTACCGCGGCTGCTGG-3’) (Muyzer et al. 1995, Bakke et al. 2011). Amplification was carried out under conditions previously described in Bueche et al. (2013). Reactions were carried out in a final volume of 10 µl with 5 µl Rotor-Gene SYBR green PCR master mix (Qiagen, Germany) and 2 ng of DNA template. The qPCR negative control was water. All reactions were performed in triplicate.

In order to verify the colonization and persistence of YD4 in the rhizosphere, a quantification of a region in the 16S gene previously found to be specific for the endophyte *Enterobacter radicincitans* (*Kosakonia radicincitans* DSM 16656^T) was performed (Ruppel et al. 2006). The degenerated 519f primer proposed by Ruppel et al. (2006) was modified to match specifically the nucleotide sequence of YD4 (5’-CAGCAGCCGCGGTAATAC-3’). The reverse primer and the TaqMan probe were used as proposed previously (Ruppel et al. 2006). Reactions were carried out in a final volume of 25 µl with 300 nM 519f-YD4 primer and *E. radicincitans* reverse primer, 100 nM *E. radicincitans* TaqManTM probe and 2 ng of DNA template. The mastermix used was the QuantiTectTMProbe PCR Kit (Qiagen, Germany). DNA extracted from YD4 and from *Desulfotomaculum reducens* were used as positive and negative controls, respectively. The qPCR negative control was water. All reactions were performed in triplicate. Annealing time was modified to 57°C, following a five-cycle touchdown step starting from 61°C, in order to enhance specific YD4 amplification in the samples.

The standard curves for quantification of both V3 and *K. radicincitans* YD4 specific 16S rRNA gene regions, were prepared from 10-fold dilutions (10^2 to 10^8 copies/µl) of a plasmid in which the entire 16S rRNA gene of YD4 was inserted. The TOPO TA cloning kit (Invitrogen, California) was used to produce this plasmid in One Shot TOP10F’ chemically competent *E. coli* cells (Invitrogen, California), following the manufacturer’s guidelines. Plasmid DNA was extracted with the Wizard Plus SV Miniprep DNA purification system (Promega, Wisconsin) following the manufacturer’s instructions. DNA quantification was carried out with a Qubit 2.0 fluorometer and assay kit (Invitrogen, California) and the number of gene copies was calculated based on this quantification.

Design of a novel strain-specific PCR quantification approach

Using the online platform of EDGAR (Blom et al. 2009) a comparative analysis among YD4 genome and eleven closely related available genomes (*K. radicincitans* DSM 16656^T, *Kosakonia radicincitans* UNENT01/12, *Enterobacter aerogenes* EA1509E, *Enterobacter asburiae* LF7a, *Enterobacter cloacae* EcWSU1, *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^T, *Enterobacter cloacae* subsp. *cloacae* ENHKU01, *Enterobacter cloacae* subsp. *cloacae* NCTC 9394*, Enterobacter cloacae* subsp. *dissolvens* SDM, *Enterobacter* sp. 368 and *Enterobacter* sp. SST3) revealed 218 unique CDSs in the strain YD4 (Supplementary Table 1). The verification of these 128 singletons was performed by a NCBI Blast search against the nr database of GenBank. A candidate gene encoding for a hypothetical protein (LG58_6128) was selected to design a putative strain-specific primer set for YD4 with the Primer-Blast tool from NCBI (Ye et al 2012). The primer set obtained was YD4f (5’ TGCTACATCGGGCTATTTTTCG 3’) and YD4r (5’ TTGCAATAATTACGCGTTTACCTCT 3’). Its specificity was confirmed by a NCBI Blast search in the GenBank database and by performing a PCR amplification using closely related bacterial strains: *Enterobacter cloacae* Neu 1000, *E.*

cloacae Neu 1027, *Enterobacter aerogenes* Neu 1036; and strains isolated from Yerba Mate rhizosphere: *Rhizobium pusense* YP3, *Pseudomonas putida* YP2, *Sphingobium yanoikuyae* YD3, *Rhizobium cellulosilyticum* YP1, *Acinetobacter radioresistens* YD1, and *Ensifer adhaerens* YN2 (Collection, University of Neuchâtel).

4.4. Results and discussion

Plant growth promoting effect of K. radicincitans YD4

K. radicincitans YD4 presented a positive effect on the growth of Yerba Mate seedlings cultivated in soil under nursery conditions (Figure 1). The bacterial inoculant increased by 54% the dry shoot and by 120% the dry root weights of inoculated seedlings in comparison to un-inoculated controls. These differences were statistically significant. No significant increase in the concentration of N, P, K, Mg and Ca in shoots accompanied the increase of biomass observed in inoculated seedlings in comparisons to the controls (Table 1). These results confirm the potential of YD4 as a bio-inoculant to increase the biomass yields of seedlings cultivated in nursery as observed in a previous study (Bergottini et al. 2015).

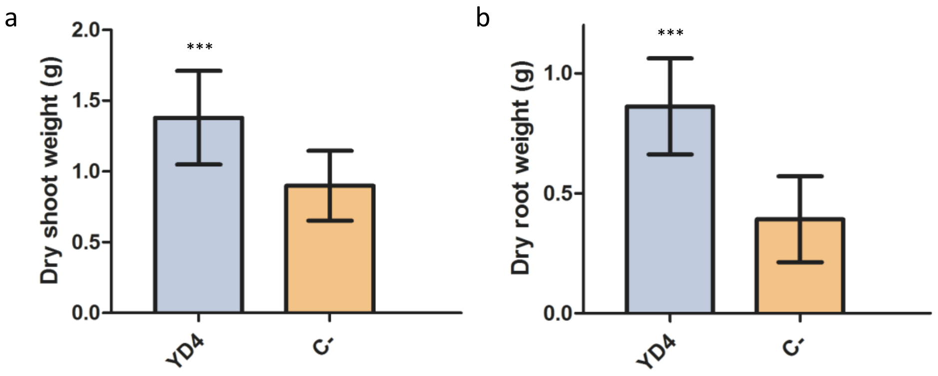

Figure 1. Effect of bio-inoculation with the PGPR strain *Kosakonia radicincitans* YD4 on the growth of Yerba Mate seedlings in nursery. Means of shoot dry weight (a) and root dry weight (b) were calculated from 50 plants per treatment after 2 months of cultivation. The performance of the bacterial inoculant and the un-inoculated control were compared with Wilcoxon test. Statistical differences between treatments are shown as: *** P ≤ 0.001.

Table 1. Macronutrients content of inoculated and control seedlings

Treatment	Macronutrients in shoot (%)				
	N	P	K	Ca	Mg
YD4	1.06 ± 0.321	0.342 ± 0.054	1.062 ± 0.243	0.938 ± 0.062	0.828 ± 0.082
C-	1.05 ± 0.343	0.378 ± 0.093	0.686 ± 0.150	1.18 ± 0.148	0.74 ± 0.025

Tests on the specificity of the PCR quantification assay

Due to the fact that the 16S rRNA gene is universal to prokaryotes, prior to the use of the primers in the rhizosphere, a specificity control test of the primers and probe was performed. For this, the endospore-forming bacterium *D. reducens* (Collection University of Neuchâtel) was selected because it is not a closely relative to YD4, and therefore was expected to yield no amplification or values close to the threshold of noise detection. Using the annealing conditions proposed in the original publication reporting the design and use of the specific qPCR method for *K. radicincitans* DSM 16656^T (Ruppel et al. 2006), we observed unspecific amplification in the order of 10^5 gene copies/ng of DNA in the negative control strain (Table 2). Although, based on the same initial concentration of DNA, the level of detection of YD4 was one order of magnitude higher (10^6), the level of unspecific amplification was unacceptable for working with the published method in the rhizosphere samples.

Therefore, a second series of amplification was attempted increasing the annealing temperature to boost specificity (Table 2). In the touchdown method, the level of detection of YD4 remained in the order of magnitude of 10^6 gene copies/ng of DNA. For *D. reducens* quantification decreased to the order of magnitude of 10^4 gene copies/ng of DNA, but this value is still far from the noise level of the method (10^2 gene copies).

Based on the results of our specificity test, it is highly likely that the primers and probe designed to be species-specific for a region of the 16S rRNA gene of *K. radicincitans* DSM 16656^T (Ruppel et al. 2006) will fail to fulfill this purpose in rhizospheric soil. Although the authors confirmed the specificity of the primers using the following bacterial strains (*Azospirillum* sp. A246, *Azospirillum irakense* DSM 11586, *Bacillus subtilis* DSM 15029, *Butyrivibrio fibrisolvens* DSM 3071, *Clostridium thermocellum* DSM 1237, *Dictyoglomus thermophilum* DSM 3960, *Herbaspirillum* spp. Accession No. AF364861, *Rhodobacter capsulatus* DSM 1710, *Rhodothermus marinus* DSM 4252, *Ruminococcus albus* DSM20455, *Sporosarcina pasteurii* DSM 33, *Stigmatella aurantiaca* DSM 1035, *Vibrio proteolyticus* DSM30189, CC322, K27, W7/15, CC307 (bacterial isolates from roots and soil of the authors), and the archaeal strain *Methanococcus voltae* DSM 1537) (Ruppel et al. 2006), they failed with our *D. reducens* strain.

Table 2. Specificity control test of the strain-specific qPCR assay

	Annealing T°	
Strain	53.2°C (Ruppel et al. 2006)	57°C (touchdown)
YD4	3,09E+06	3,90E+06
D. reducens	3,52E+05	9,43E+04

PCR quantification in the rhizosphere

The results represented in the figure 2 reflect the low-specificity of the primers and probe to track the bacterial inoculant YD4 in the rhizosphere. For instance, we expected to observe at least a higher pseudo-specific detection of YD4 in the rhizosphere of inoculated seedlings (BI-YD4) within the day of inoculation (day 0). However, both inoculated (BI-YD4) and un-inoculated control (C-YD4) seedlings presented similar pseudo-specific YD4 detection during the first week. A shift in YD4 detection occurred at the 3rd week in comparison to un-inoculated controls, however we are not able to associate this to our bacterial inoculant.

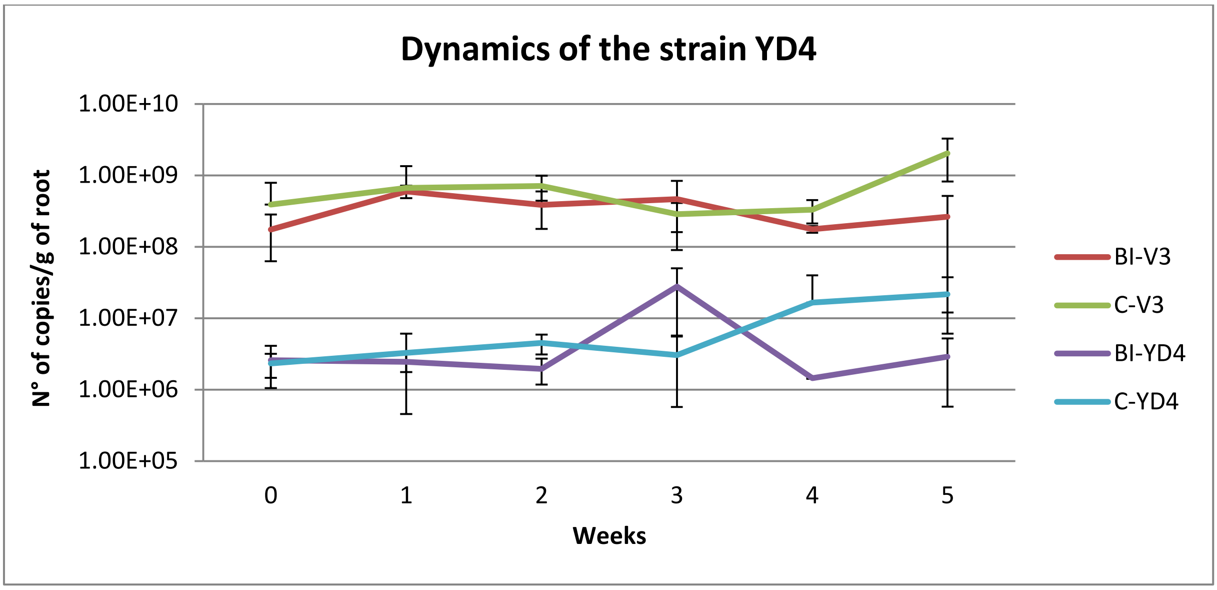

Figure 2. Quantification of *K. radicincitans* YD4 and total bacteria in the rhizosphere of inoculated and control Yerba Mate seedlings using a pseudo-specific qPCR approach.

PCR quantifiation in the rhizosphere using a nobel primet set

The dynamics of the introduced bacterial inoculant YD4 was analyzed using the strain-specific primer set YD4f/YD4r from the day of inoculation (day 0) until 5 weeks post inoculation in the rhizosphere of inoculated seedlings (BI-YD4) and non-inoculated control seedlings (C-YD4) (Figure 3). At the day of inoculation (Day 0) a higher detection of the strain YD4 (2 orders of magnitude higher) was observed in inoculated seedlings (BI-YD4) in comparison to non-inoculated controls (C-YD4). Afterwards, a rapid decrease in the detection of YD4 (BI-YD4) occurred (from 4.75 x 10^6 copies/g at the day 0 to 5.46 x 10^5 copies/g at week 1). Then, the detection of YD4 (BI-YD4) remained similar for two weeks (week 1 to 3) until a second decreased occurred from week 3 until achieving 5.77 x 10^4 copies/g of root at the week 5. In the rhizosphere of non-inoculated seedlings (C-YD4) a detection of native bacterial populations potentially related to *K. radicincitans* YD4 was observed however with a lower number of copies in comparison to inoculated seedlings.

According to these results, we are able to demonstrate that the bacterial inoculant YD4 colonizes and persists in the rhizosphere of Yerba Mate seedlings after its application. Since the day of inoculation until 5 weeks the strain YD4 was detected in the rhizosphere with a detection of 5.77 x 10^4 copies/g of root at the end of the experiment.

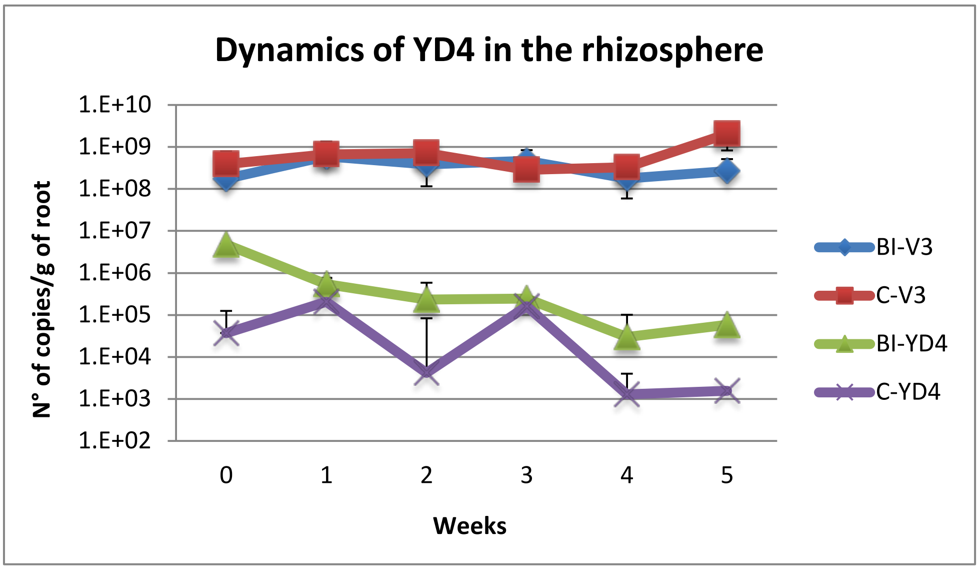

Figure 3. Quantification of *K. radicincitans* YD4 and total bacteria in the rhizosphere of inoculated and control Yerba Mate seedlings using a strain-specific qPCR approach.

4.5. Conclusion

This study evaluated the prevalence of the bio-inoculant YD4 in the rhizosphere soil of Yerba Mate seedlings using a strain-specific qPCR approach. The analysis of the dynamics of the bio-inoculant strain YD4 showed that after its application in soil, YD4 is able to colonize and persist within the rhizosphere at least for 5 weeks (time analyzed in this study). These results allow to attribute the plant growth-promoting effect observed in seedlings with the presence of the introduced bacterial inoculant YD4. In addition, this study provides a specific approach to track this bacterial inoculant in soil, a requisite for selecting a bacterial inoculant for biotechnological applications.

4.6. Acknowledgments

This work was supported by the Swiss National Science Foundation grant No. 31003A_152972 (T. Junier), Fondation Pierre Mercier pour la science (S. Filippidou). The field work was supported by the Laboratory of Microbiology, University of Neuchâtel to V.M. Bergottini. We are very thankful for the valuable collaboration of Marcelo Stockar, Silvana Sawostjanik and Lucrecia Barchuk.

Supplementary table 1. Singletons of *K. radicincitans* YD4 calculated in comparison to 11 closest genomes available in the EDGAR platform.

1	isochorismatase family protein	31	putative endochitinase	61	hypothetical protein	91	hypothetical protein
2	putative membrane protein	32	hypothetical protein	62	bacterial transcriptional regulator family protein	92	hypothetical protein
3	major Facilitator Superfamily protein	33	hypothetical protein	63	major Facilitator Superfamily protein	93	polysaccharide biosynthesis family protein
4	doxX-like family protein	34	hypothetical protein	64	bacterial Ig-like domain family protein	94	hypothetical protein
5	hypothetical protein	35	hypothetical protein	65	hypothetical protein	95	hypothetical protein
6	bacterial regulatory s, tetR family protein	36	hypothetical protein	66	hypothetical protein	96	hypothetical protein
7	hypothetical protein	37	hypothetical protein	67	hypothetical protein	97	hypothetical protein
8	restriction endonuclease family protein	38	catalase family protein	68	bacterial regulatory s, tetR family protein	98	hypothetical protein
9	nmrA-like family protein	39	hypothetical protein	69	isochorismatase family protein	99	hypothetical protein
10	hypothetical protein	40	hypothetical protein	70	maoC like domain protein	100	hypothetical protein
11	hypothetical protein	41	hypothetical protein	71	immunity 45 family protein	101	hypothetical protein
12	HD domain protein	42	hypothetical protein	72	hypothetical protein	102	glyoxalase/Bleomycin resistance
13	hypothetical protein	43	hypothetical protein	73	hypothetical protein	103	hypothetical protein
14	bacterial regulatory s, tetR family protein	44	hypothetical protein	74	hypothetical protein	104	hypothetical protein
15	hypothetical protein	45	hypothetical protein	75	hypothetical protein	105	hypothetical protein
16	mce related family protein	46	hypothetical protein	76	hypothetical protein	106	putative membrane protein
17	paraquat-inducible A family protein	47	hypothetical protein	77	bacterial extracellular solute-binding s, 5 Middle family protein	107	hypothetical protein
18	hypothetical protein	48	hypothetical protein	78	beta subunit of N-acylethanolamine-hydrolyzing acid amidase family protein	108	phage tail family protein
19	hypothetical protein	49	hypothetical protein	79	alpha/beta hydrolase family protein	109	hypothetical protein
20	putative membrane protein	50	hypothetical protein	80	bacterial regulatory s, gntR family protein	110	hypothetical protein
21	putative membrane protein	51	putative dephospho-CoA kinase	81	bacterial regulatory helix-turn-helix, lysR family protein	111	hypothetical protein
22	hypothetical protein	52	helix-turn-helix family protein	82	D-alanyl-D-alanine carboxypeptidase family protein	112	hypothetical protein
23	putative lipoprotein	53	carbon-nitrogen hydrolase family protein	83	putative lipoprotein	113	clp protease family protein
24	bacterial regulatory helix-turn-helix, lysR family protein	54	binding--dependent transport system inner membrane component family protein	84	putative lipoprotein	114	hypothetical protein
25	NAD binding domain of 6-phosphogluconate dehydrogenase family protein	55	NMT1-like family protein	85	hypothetical protein	115	hypothetical protein
26	hypothetical protein	56	hypothetical protein	86	hypothetical protein	116	hypothetical protein
27	hypothetical protein	57	amidase family protein	87	putative membrane protein	117	single-strand binding family protein
28	hypothetical protein	58	eamA-like transporter family protein	88	hypothetical protein	118	hypothetical protein
29	hypothetical protein	59	AAA domain protein	89	hypothetical protein	119	hypothetical protein
30	hypothetical protein	60	hypothetical protein	90	hypothetical protein	120	hypothetical protein

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Chapter 5: Community structure and diversity of the root-associated microbiome of Yerba Mate

VM Bergottini, V Hervé, DA Sosa, P Junier

5.1. Summary

The diverse bacterial and fungal taxa inhabiting plant roots can play a role in modulating plant nutrition and health. Hence, a deeper understanding of the root-associated microbiota can guide the isolation and selection of microorganisms with potential plant growth promoting activities. In this study we analyzed the diversity and community composition of the bacterial and fungal microbiomes associated to the rhizoplane and endosphere of Yerba Mate (*Ilex paraguariensis* St. Hill.). Yerba Mate is an important crop in South America for which a decrease in yield associated to unsustainable agricultural practices has been observed. Eleven plantations with different historical agricultural practices located across the state Misiones, the principal productive region of this crop, were selected for analysis. Microbial alpha diversity was neither affected by the agricultural management nor by the geographical location of the plantations. Largely, *Proteobacteria* dominated the bacterial community composition in all plantations. A shift towards the dominance of *Firmicutes* in one medium-productive plantation was associated to soil compaction, while a shift in *Glomeromycota* in a low-productive plantation was related to a potential selective pressure of the tree for mycorrhizal associations. In the limited geographical area studied, location contributed to structuring the bacterial community composition, whereas location and soil pH were the driving factors to structure the fungal communities. Two OTUs assigned to *Acidibacter-like* and *Fusarium oxysporum* were present in all the samples. Regarding potential plant growth promoters, *Burkholderia, Enterobacter* and *Acaulospora* can be considered as the bacterial and mycorrhizal candidates for further isolation and selection. Our study provides the first description of the root-associated microbiota of Yerba Mate and constitutes a stepping-stone towards harnessing the role of microbes in the sustainable cultivation of this crop.

5.2. Introduction

The problematic of land degradation affects seriously the production of Yerba Mate in Misiones province, northeast Argentina (INTA-INYM 2008), the leading productive region of this crop. Yerba Mate (*Ilex paraguariensis* St. Hill.), Aquifoliaceae, is a native tree from northeastern Argentina, Paraguay, South Brazil and part of Uruguay (Grondona 1954). The leaves of Yerba Mate are used for producing a healthy and energizing beverage widely consumed as an alternative to coffee. Aside South America, Yerba Mate tea is becoming increasingly popular in others markets, including the Middle East, Europe, and the United States, where it is appreciated as a natural energizing drink due to its high content of antioxidants and nutritional benefits (Heck and De Mejia 2007). Currently, an important area of Yerba Mate production is in a process of degradation due to inadequate agricultural practices maintained over time (INTA-INYM 2008). In order to maintain and improve soil quality, more sustainable agricultural practices have been implemented in Yerba Mate agricultural systems, including zero-tillage, use of green covers and associative trees (Burtnik 2003, Day et al. 2010, Ilany et al. 2010).

In agricultural soils, the soil microbiota associated to plant roots play a key role in plant growth through the production of phytohormones (Spaepen and Vanderleyden 2011) or the facilitation of inaccessible mineral nutrients (Rodrigues et al. 2008). In other cases, the soil microbiota provides plant protection by exerting an antagonism effect against phytopathogens (Compant et al. 2005). Currently, high-throughput DNA sequencing approaches are used to analyze the root-associated microbiome of different plant species in order to reveal taxonomic and functional microbes involved in plant growth promotion (Uroz 2010, Gottel et al. 2011). In a recent study, the isolation and selection of diazotrophic bacteria associated with sugarcane was based on the enriched taxa present in the roots of this crop (Paungfoo -Lonhienne et al. 2014).

The root-associated microbiome of Yerba Mate remains unexplored and its analysis might as well constitute a guide for the isolation and selection of particular microbial taxa contributing to plant nutrition and protection particualrly in low-input fields. In a previous study, it has been shown that bio-inoculation with plant growth promoting rhizobacteria isolated from Yerba Mate roots increased the biomass yields of seedlings in nursery (Bergottini et al. 2015). In addition, endomycorrhizal associations in this crop have been reported in native trees of the Atlantic forest (Andrade et al. 2000) and in seedlings cultivated in nurseries (Gaiad and Lopez 1986). The analysis of the root mirobiome of Yerba Mate will allow to identify the enriched bacterial and fungal taxa associated to this crop that could be exploited for biotechnological purpouses. In these context, we hypothesize that the root microbiome of Yerba Mate may present microbial taxa involved in functions related to plant growth promotion and protection. According to this, the aim of this study was to analyse the root-associated microbiome of Yerba Mate in plantations with different agricultural management practices located at different sites within the province of Misiones, the main productive area of this crop in South America. The communitiy composition, the genetic and phylogentic diversity of the root microbiome was compared between plantation sites and cultivation practices to address the major factors contributing to structure the root microbiome in this crop.

5.3. Material and Methods

Roots collection and soil sample analysis.

Eleven plantations located across the principal region of Yerba Mate production in northeast Argentina were selected to analyze the root-associated microbiome of Yerba Mate (Supplementary figure 1). The selected plantations presented different historical agricultural management practices and contrasting productivity yields. A detailed description of each plantation is given in Table 1. In each plantation seven plants were randomly selected to collect 3 subsamples of roots (up to a depth of 10 cm) per plant and pooled to constitute one sample per plantation. Bulk soil samples were collected to determine extractable phosphorus (Bray and Kurtz 1945), nitrogen (by the semi-micro-Kjeldahl method) (Kjeldahl

1833); and potassium (K), sodium (Na), magnesium (Mg), and calcium (Ca) (measured in an ammonium acetate extract). The soil chemical properties are described in Table 2. The sampling was held in the winter season of July 2013.

DNA extraction, PCR and pyrosequencing

To obtain the root-associated microbiome, roots were washed with sterile distillated water (under laminar flow hood) in order to remove the rhizospheric soil fraction not closely attached to Yerba Mate roots. One gram of washed roots per sample was processed for DNA extraction with the FastDNA Spin Kit for Soil (MP Biomedicals, California). Amplicon generation and further 454-pyrosequencing analysis was performed by Eurofins Genomics GmbH (Switzerland). To target the bacterial communities the 16S rRNA gene was amplified with a primer set 27F (Lane 1991) and 1492R (Stackebrandt and Liesack 1993) whereas the fungal communities were targeted by amplifying the ITS region with the primer set ITS1-F/ITS4 (White et al. 1990, Gardes and Bruns 1993). The sequencing of the 16S rRNA and ITS was performed unidirectional using the forward primers.

Sequence processing

Bacterial and fungal amplicon sequences were analyzed independently, using the mothur software version 1.34.4 (Schloss et al. 2009). Bacterial reads were processed by largely following the Schloss standard operating procedure (Schloss et al. 2011). First, sequencing errors were reduced by implementation of the AmpliconNoise algorithm and low-quality sequences were removed (minimum length of 360 bp, allowing 1 mismatch to the barcode, 2 mismatches to the primer, and homopolymers no longer than 8 bp). Sequences were then trimmed to keep only high quality reads ($Q \geq 35$). Barcode and primer sequences were removed. Subsequently sequences were aligned to the SILVA reference database release 119 (Quast et al. 2013) and preclustered (pre.cluster, diffs=1). Chimeras were removed using the chimera.uchime mothur command and singletons were excluded. Finally, sequences were classified using the naïve Bayesian classifier (Wang et al. 2007) implemented in mothur with the SILVA reference database release 119 (Quast et al. 2013). Operational taxonomic units (OTUs) were generated using the average neighbor algorithm. An OTU was defined at the 97% sequence similarity level. Sequences identified as chloroplasts or mitochondria were removed.

Regarding the fungal sequences, reads were quality processed with the same parameters as described above except that the minimum length was set to 370 bp. After removing chimera and singletons, the presence of fungal ITS was checked using ITSx version 1.0.11 (Bengtsson-Palme et al. 2013) and non-ITS sequences were discarded. Subsequently ITS sequences were pairwise aligned to generate a distance matrix using the pairwise.seqs command. Finally, sequences were classified using the naïve Bayesian classifier (Wang et al. 2007) implemented in mothur with the UNITE v6_sh_dynamic database (Kõljalg et al. 2013).

Operational taxonomic units (OTUs) were generated using the average neighbor algorithm. An OTU was defined at the 97% sequence similarity level.

Diversity and statistical analysis

Rarefaction curves, diversity indices (both calculated from 10000 iterations) and relative abundances of OTUs were estimated using mothur (Schloss et al., 2009). All statistical analyses were computed using R software version 3.1.3 (R Development Core Team, 2015). Comparisons of both the alpha diversities and soil chemical properties according to geographical location or the plant productivity were performed using non-parametric Kruskal-Wallis tests. After normalization by random subsampling (1376 and 4785 sequences in each sample for the bacterial and fungal community, respectively, corresponding to the lowest number of sequences in a sample), OTU matrices were log (1+x) transformed and Bray-Curtis dissimilarity matrices were computed for both bacterial and fungal communities using the *vegan* package (Oksanen et al., 2012). Global Non-Metric MultiDimensional Scaling (GNMDS) based on Bray-Curtis distance were computed using the same package. The effects of soil chemical properties and geographical location on both the bacterial and fungal community composition were estimated by a distance-based permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) using “adonis” function of the *vegan* package with Bray-Curtis distance matrix and 100000 random permutations.

The genetic diversity between the different locations was evaluated using Analysis of Molecular Variance (AMOVA) and Homogeneity of Molecular Variance (HOMOVA), both based on 10^6 iterations and computed with mothur. For the bacterial communities, the effect of the location on the phylogenetic diversity was investigated based on weighted UniFrac distances (Lozupone & Knight, 2005). Bacterial 16S rRNA gene sequences aligned in Mothur were used to compute a neighbor joining phylogenetic tree with Clearcut (Sheneman *et al.* 2006). Subsequently, this tree was used to compute a phylogenetic distance matrix based on the unweighted UniFrac algorithm implemented in Mothur (Lozupone & Knight, 2005).

The taxonomic composition of different core microbiomes was also investigated. A core microbiome was defined here as a set of OTUs present within all samples of a group and with a relative abundance >1%.

5.4. Results and discussion

Soil properties of the plantations

Eleven plantations with different historical management practices and productivity yields located in two regions of the state of Misiones were selected to collect samples of Yerba Mate roots (Table 1) (Supplementary Figure 1). The soil chemical properties were analyzed in each plantation (Table 2) in order to reveal the nutritional status of soils long-term managed under different agricultural practices. Because of the lack of replicates, no

Table 1. Agricultural management of the plantations selected to investigate the diversity of the root-associated bacterial and fungal communities of Yerba Mate

Plantation location #	**Latitude**	**Longitude**	**Productivity ***	**P fertilization (kg/ha)**	**N fertilization (kg/ha)**	**Agricultural management**	**Age of plants**
AI1	25°49'58.80''S	53°55'56.32''W	Medium	15	72	Monoculture, tillage-zero, green covers	25
AI2	25°50'1.28"S	53°.55'56.00"W	High	25	125 - 150	Monoculture, tillage-zero, green covers	25
AI3	25°50'4.25"S	53°55'54.76"W	High	25	125 - 150	Monoculture, tillage-zero, green covers	25
AL	25°47'40.15"S	53°58'26.69"W	Low	0	0	Monoculture, conventional tillage, no fertilization, no green covers	25
AM	25°50'1.21"S	53°56'8.87"W	Medium	15	72	Rainforest converted into an agroforestry system with native tree, tillage-zero, green covers	25
AA	25°50'0.76"S	53°55'51.11''W	Medium	15	72	Co-cultivated with few tree species, native green covers	25
JA1	26°59'32.67"S	55°14'01.68"W	Medium	15	72	Monoculture, tillage-zero, green covers	25
JA2	26°59'35.43"S	55°14'2.15"W	High	25	125 - 150	Monoculture, tillage-zero, green covers	25
JA3	26°59'34.75"S	55°14'0.96"W	High	25	125 - 150	Monoculture, tillage-zero, green covers	25
SPB	27°8'18.07''S	55°25'27.11''W	Low	10	50	Monoculture, conventional tillage, fertilization, no green covers	30
SPA	27°6'10.96''S	55°18'55.26''W	Medium	10	75	Co-cultivated with few tree species, native green covers	30

*High: 16.000-18.000 kg/ha, Medium: 13.000 kg/ha, Low: 7.000 kg/ha

[#] Plantation location: Andresito (A), Jardin America (JA), Santo Pipo (SP)

Table 2. Chemical properties of soils.

Plantation	pH	OM %	C %	N %	C/N	P mg kg^{-1}	K mg kg^{-1}	Ca mg kg^{-1}	Mg mg kg^{-1}	Na mg kg^{-1}
AI1	4.14	4.24	1.89	0.21	10.61	6.38	136.9	695.4	185.9	20.7
AI2	5.06	4.09	1.82	0.25	9.46	4.29	113.4	801.6	193.2	18.4
AI3	6.52	3.22	1.44	0.21	8.66	5.33	223	1567	143.4	16.1
AL	5.19	3.37	1.5	0.25	8.29	3.59	109.5	847.6	178.6	16.1
AM	6	5.95	2.66	0.43	6.39	1.62	117.3	1508.9	335.4	20.7
AA	5.18	4.68	2.09	0.21	10	4.64	nd	nd	nd	nd
JA1	4.65	3.84	1.71	0.27	8.29	8.58	74.3	565.1	143.4	16.1
JA2	4	2.9	1.29	0.2	8.4	5.22	35.2	212.4	35.2	18.4
JA3	3.96	3.73	1.66	0.25	8.67	60.42	101.7	470.9	79	23
SPB	4.63	5.89	2.63	0.32	10.78	81.87	nd	nd	nd	nd
SPA	5.77	4.19	1.87	0.28	7.43	2.67	195.5	2192.3	200.5	12.2

nd: not determined

statistical analysis could be performed between soil plantations. However, soil properties could be compared between the northern and southern regions. No significant differences were observed in edaphic variables such as pH, OM, C, N and P between these two regions (Kruskal-Wallis rank sum *p*>0.05).

Pyrosequencing data and OTUs assembling

A total of 106620 raw pyrotag reads were obtained from 11 samples for the bacterial diversity analysis. After quality control (chimera, singletons, and chloroplast/mitochondrial sequences removal) 29470 reads were retained with an average length of 354 bp. Sequences were clustered in 1566 bacterial operational taxonomic units (OTUs) at a sequence similarity of 97%. To compare the diversity among samples with different read counts, we normalized the data by selecting the lowest number of sequences in a sample (1376) to rarefy the data set. A total of 15136 sequences were obtained corresponding to 1228 OTUs. For the fungal diversity analysis, a total of 247048 raw pyrotag reads were obtained from the 11 samples. After chimera and singletons removal, 172583 reads remained with an average length of 398 bp, including 3242 unique sequences. This corresponded to 1061 fungal OTUs at 97% similarity level. After normalization by random subsampling (4785 sequences by sample), 52420 sequences corresponding to 907 OTUs were obtained. For both data sets the Good’s coverage estimation (Good 1953) (Table 3) and the rarefaction curves (Figure 1) showed that the sequencing depth was sufficient to estimate and compare the microbial diversity of the samples.

Alpha-diversity and taxonomic composition

We analyzed if either the geographical location (north vs south plantations) or the agricultural practices of the plantations have an effect on the alpha diversity of bacterial and/or fungal communities. No significant effects of the location or of the agricultural practices were observed in the number of OTUs (after normalization), Chao estimation (richness), Shannon diversity and Shannon evenness indices ($p>0.05$, Kruskal-Wallis rank sum test) (Table 3). Most of the samples presented a similar alpha diversity for the bacterial and fungal communities, except the bacterial community of AM that presented a higher number of OTUs (Figure 1a) supported by a higher Chao estimation of richness (Table 3). A different historical agricultural management (Table 1) might explain the different behavior of the bacterial community in the sample from the AM plantation. This plantation was established in a logged-rainforest field and has been managed from its creation as an agroforestry system with native trees. In a previous study, an increase in the microbial alpha diversity occurred in forest-soils converted into agricultural fields (Rodrigues et al. 2013), which might contribute to a higher bacterial richness in the AM plantation.

At the phylum level, *Proteobacteria* (57%), *Acidobacteria* (13%), *Firmicutes* (8%), *Actinobacteria* (7%) and *Plantomycetes* (5%), composed the rarefied bacterial root-associated microbiome of Yerba Mate. Other phyla including *Chloroflexi*, *Bacteriodetes* and *Verrumicrobia* were represented at a lower relative abundance (2%), while the rest of the phyla represented 1% or less of the community composition. The overall bacterial composition tended to be similar in plantations, regardless of the management practices. These results are consistent with the characterization of the root-associated microbiome of some woody species such as poplar (Gottel et al. 2011) and oak (Uroz et al. 2010), in which *Proteobacteria and Acidobacteria* were the dominating phyla. In the AA plantation, *Firmicutes* represented 63% of the total relative abundance thus breaking the stable pattern of community composition at phylum level (Figure 2a). Since the most abundant OTU was assigned to *Weissella,* a facultative anaerobe, we hypothesize that this shift in *Firmicutes* could be related to the problematic of soil compaction in this plantation since in a previous study some members of this phylum were favored in compacted soils due to their capability of anaerobic respiration (Hartmann et al. 2014).

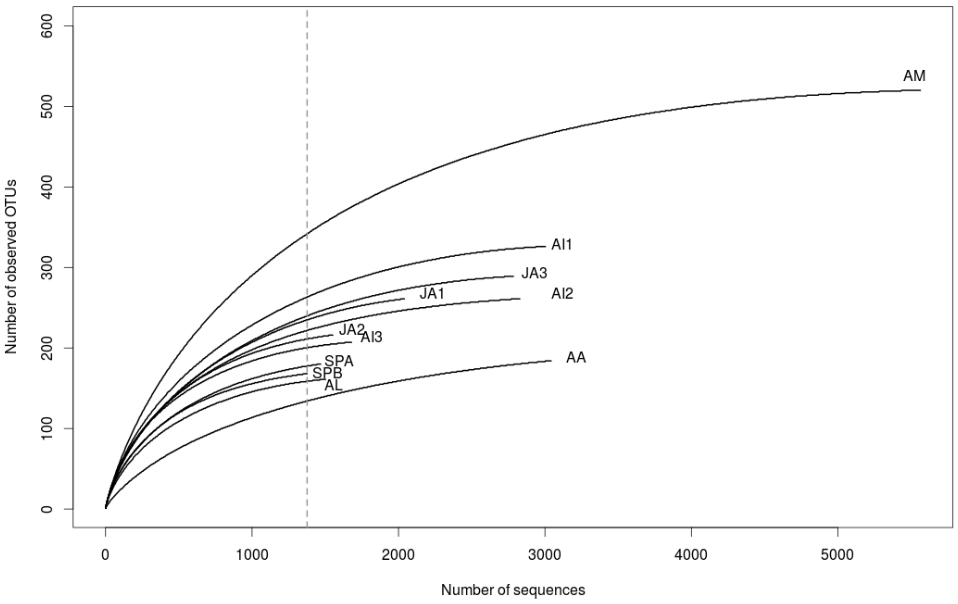
A deeper taxonomic analysis at the genera level revealed that the root-associated bacterial microbiome is mainly dominated by unclassified bacterial species, representing approximately 21% of the total OTUs genera. Among the unclassified OTUs, uncultured bacteria from the phylum *Acidobacteria* are the most abundant. However the ecological function of this group in the rhizosphere remains unknown since no isolates have been obtained from the rhizosphere so far (Nunes da Rocha et al. 2009).

For those OTUs that could be classified, we observed a decreasing relative abundance of the following genera: *Burkholderia* (17%), *Acidibacter-like* (8%), *Weissella* (6%), *Bradyrhizobium* (6%), *Phenylobacterium* (3%), *Enterobacter* (3%), *Gemmata* (2%), *Bryobacter* (2%), *Mycobacterium* (2%) and *Rhizobium* (2%). Many species of *Burkholderia* are known to be enriched in the rhizosphere of a wide range of plant species, including sugarcane (Paungfoo-Lonhienne et al. 2014), oak (Uroz et al. 2010) and pine (Timonen et al. 2006). Indeed, this genus is known to present a particular wide tolerance to acidic soils (Stopnisek et al. 2013) and many species have been reported as plant growth promoting bacteria (Suárez-Moreno et al. 2012). Among the other abundant genera that could play a role in plant growth and protection are the lactic acid bacteria *Weissella* reported to present antagonistic activities against plant pathogenic bacteria and fungi (Fhoula et al. 2013). In addition, *Bradyrhizobium* and *Rhizobium* reported as potential PGPR for non-legumes (Antoun et al. 1998), and *Enterobacter* reported as phosphate solubilizing bacteria associated with Yerba Mate rhizosphere (Collavino et al. 2010), could play a role in contributing to plant nutrition. The occurrence of *Phenylobacterium* might be related to the application of herbicides in these plantations since this genus can degrade the herbicide chloridazon (Lingens et al. 1985). On the other hand, the predominance of *Acidibacter-like* can be associated to the iron-rich environment of these lateritic soils and to the micro-aerophilic conditions of the rhizoplane and endosphere niches, favoring an enrichment of iron-reducing bacteria. It is worth to mention that this genus represent the most abundant OTU in the bacterial dataset reflecting a particular association with Yerba Mate roots. This bacterial OTU could not be accurately classified with the Silva database, therefore we used EzTaxon (Chun et al. 2007) to obtain a better identification (95% of similarity). Although it is well reported that plant roots exert a selective pressure in the composition and abundance of the root-associated microbiome (Uroz et al. 2010; Gottel et al. 2011; Peiffer et al. 2013), the abundance of *Acidibacter-like* in the surrounding bulk soil should be analyzed further to confirm this.

Table 3. Values of alpha diversity calculated for the bacterial and fungal communities of Yerba Mate roots.

	Bacterial α-diversity					**Fungal α-diversity**				
Plantation	Good's Coverage	Observed richness (♯ OTUs)	Chao1 Richness	Shannon Diversity	Shannon Evenness	Good's Coverage	Observed richness (♯ OTUs)	Chao1 Richness	Shannon Diversity	Shannon Evenness
AI1	0.92	276	361	4.45	0.79	0.99	116	157	2.33	0.49
AI2	0.95	224	260	4.42	0.82	0.99	108	143	2.83	0.61
AI3	0.96	203	225	4.30	0.81	0.99	165	197	3.28	0.64
AL	0.98	159	165	3.78	0.75	1.00	142	153	3.22	0.65
AA	0.95	140	214	2.12	0.43	0.99	216	229	3.75	0.70
AM	0.90	326	443	4.68	0.81	0.99	249	268	4.29	0.78
JA1	0.94	231	283	4.23	0.78	0.99	187	219	3.52	0.67
JA2	0.97	215	233	4.35	0.81	0.99	189	236	3.22	0.61
JA3	0.93	241	317	4.32	0.79	0.99	139	186	2.76	0.56
SPA	0.97	179	193	4.03	0.78	0.99	186	237	3.45	0.66
SPB	0.98	168	179	4.00	0.78	1.00	158	158	3.79	0.75

a



b

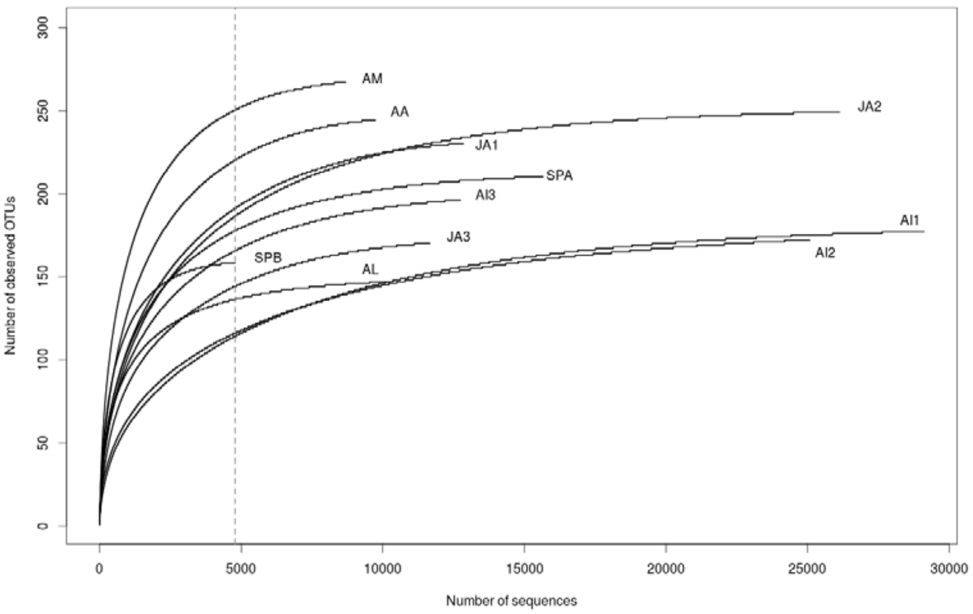


Figure 1. Rarefaction curves of the bacterial (a) and fungal (b) OTUs associated to Yerba Mate roots in eleven plantations located in the state of Misiones, Argentina. Dashed lines in each figure indicate the number of sequences selected for performing the subsampling (normalization of the data).

Ascomycota (72%), followed by *Basidiomycota* (13%), *Zygomycota* (9%) and *Glomeromycota* (5%) dominated the root-associated fungal microbiome at the phylum-level. *Chytridiomycota* and unclassified genera occurred at less than 1%. When comparing among plantations, *Ascomycota* remained dominant in all samples while *Glomeromycota* is differentially enriched in the microbiome of the sample from the AL plantation (Figure 2b). SPB and AL plantations are two fields historically managed with conventional practices reflected nowadays in low-productivity yields. However, when comparing the soil P content between these two fields, a much lower P content was measured in the AL plantation. Therefore, we hypothesize that this shift in *Glomeromycota* in AL plantation might be related to a higher selective pressure by the plant for mycorrhizae associations in soils with low P content (Mäder et al. 2000). In its natural habitat Yerba Mate is a mycorrhizal colonized tree (Andrade et al. 2000) and the taxonomical identification of these species could allow future biotechnological applications. Approximately 81% of the *Glomeromycota* OTUs belonged to unclassified genera of the family *Acaulosporeacea,* including also the most abundant mycorrhizal OTU (48% of the relative abundance of *Glomeromycota* OTUs).

Glomus sp. (9%), a group constituted by unclassified genera of *Glomeraceae* (6%), *Rhizophagus* (2%) were also identified. *Gigaspora*, *Entrophospora* and *Paraglomus* were represented in less than 1%. Approximately 2% of the OTUs could be only identified at the order level (*Glomeromycetes*). Previously, Gaiad and Lopes (1986) reported that *Acaulospora* and *Glomus* were the dominant genera colonizing Yerba Mate seedlings in different nurseries in Brazil. Since that study, there are no further reports in the literature about the mycorrhizal fungi associated to this crop. Thus this study provides the first extensive taxonomic description of Yerba Mate associated mycorrhizae.

On the other hand, the most abundant fungal genus corresponded to a group of unclassified OTUs of the order *Hysteriales* (12%). In a decreasing order of abundance we identified a group of unclassified OTUs belonging to the *Myriangiales* (9%), *Ilyonectria* (6%), *Fusarium* (5%), *Atractiellales* (5%), *Mortierella* (5%), *Acaulospora* (4%), *Cladophialophora* (4%), *Schizangiella* (4%) and *Flagellospora* (4%). Since some genera of *Hysteriaceae* (*Hysteriales*) and *Elsinoacea* (*Myriangiales*) are phytopathogens (Alexopoulus et al. 1996, Jayawardena et al. 2014), some of these OTUs could be consider as potential pathogens for this crop. The identification of these genera in the root-associated fungal microbiome of Yerba Mate could contribute to a broader understanding of potential fungal pathogens associated to this crop.

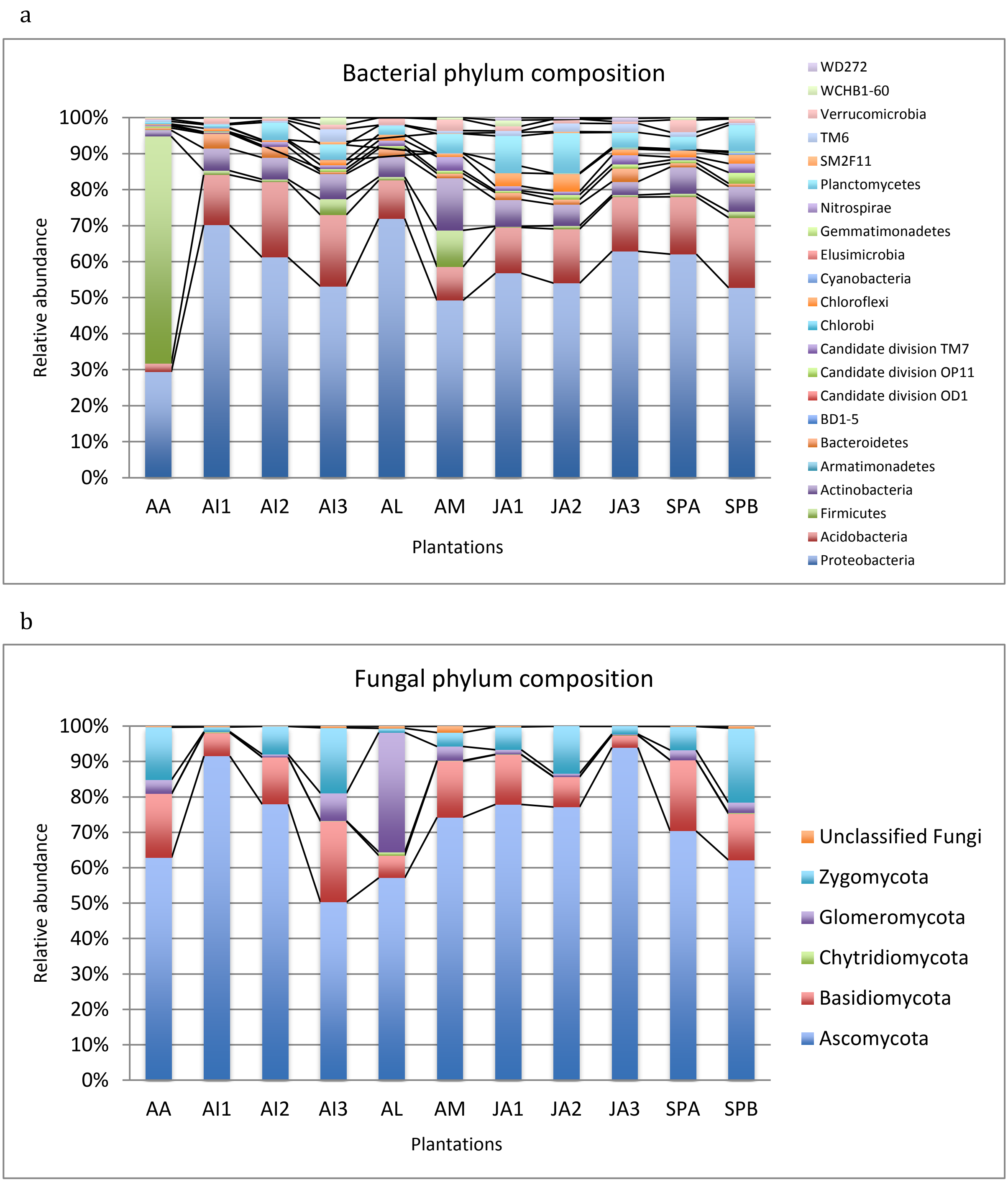


Figure 2. Bacterial (a) and fungal (b) community composition at the phylum level of Yerba Mate roots from eleven plantations.

Plantation location affects the microbial community structure

In order to evaluate if the geographical location or the agricultural practices of the plantations contributed to structure the microbial community composition among samples we performed PERMANOVA analysis. No significant differences were observed between the bacterial and fungal microbiomes of conventional and environmental-friendly plantations ($p>0.05$). However, the geographical location (northern versus southern sites) of the plantations affected significantly the bacterial community composition ($R^2 = 0.142$, $p<0.05$), the genetic diversity (HOMOVA B=0.43, $p<0.01$; AMOVA, Fs=1.76, $p<0.05$) and phylogenetic diversity (Weighted UniFrac test, W=0.23, $p<0.001$; Unweighted UniFrac test, W=0.77, $p<0.001$). If the geographical location is shaping bacterial community structure, diversity and phylogenetic diversity, we expect that closely located plantations will present similar structure. Using weighted UniFrac values as metric of community dissimilarity, we found a distance-decay relationship (Figure 4) where bacterial community dissimilarity is significantly and positively correlated with geographic distance ($R^2=0.07$, $p<0.05$). This confirmed that distant bacterial communities tend to be more dissimilar than geographically close bacterial communities associated with Yerba Mate. Ordination method also revealed that the bacterial microbiome grouped by proximity distances in the south region, where the contiguous plantations in Jardin América (JA1, JA2, JA3) and those more distant in Santo Pipo (SPL, SPA) formed a defined group (Figure 3a). However this was not the case for the plantations of Andresito in the north region (AA, AI1, AI2, AI3, AL, AM) notably dispersed in the GNMDS plot.

When comparing the fungal microbiome among samples we observed that the geographical location (PERMANOVA, $R^2 = 0.152$, $p<0.05$) and the pH (PERMANOVA, $R^2 = 0.164$, $p<0.005$) were significantly shaping the community composition. Additionally, the genetic diversity was significantly different between the two plantation locations (AMOVA, Fs=1.83, $p<0.05$). In the GNMDS plot the contiguous plantations in Jardin America were grouped together (Figure 3b). We hypothesize that their proximity and similar pH values among plantations (JA1: pH 4.65, JA2: pH 4, JA3: pH 3.96) can be the major factors contributing to the similarities on their community composition. In contrast, the more heterogeneous pH values of the contiguous plantations in Andresito (AI1: pH 4.14, AI2: pH 5.06, AI3: pH 6.52) might explain that they did not clustered together. The group constituted by some plantations from Andresito (AA: pH 5.18, AI2: pH 5.06, AI3: pH 6.52) and Santo Pipo (SPA: pH 5.77) might reflect that soil pH is contributing to determine the community composition (Figure 3b), however other unmeasured factors could drive in a major degree the community composition in those plantations dispersed on the GNMDS plot.

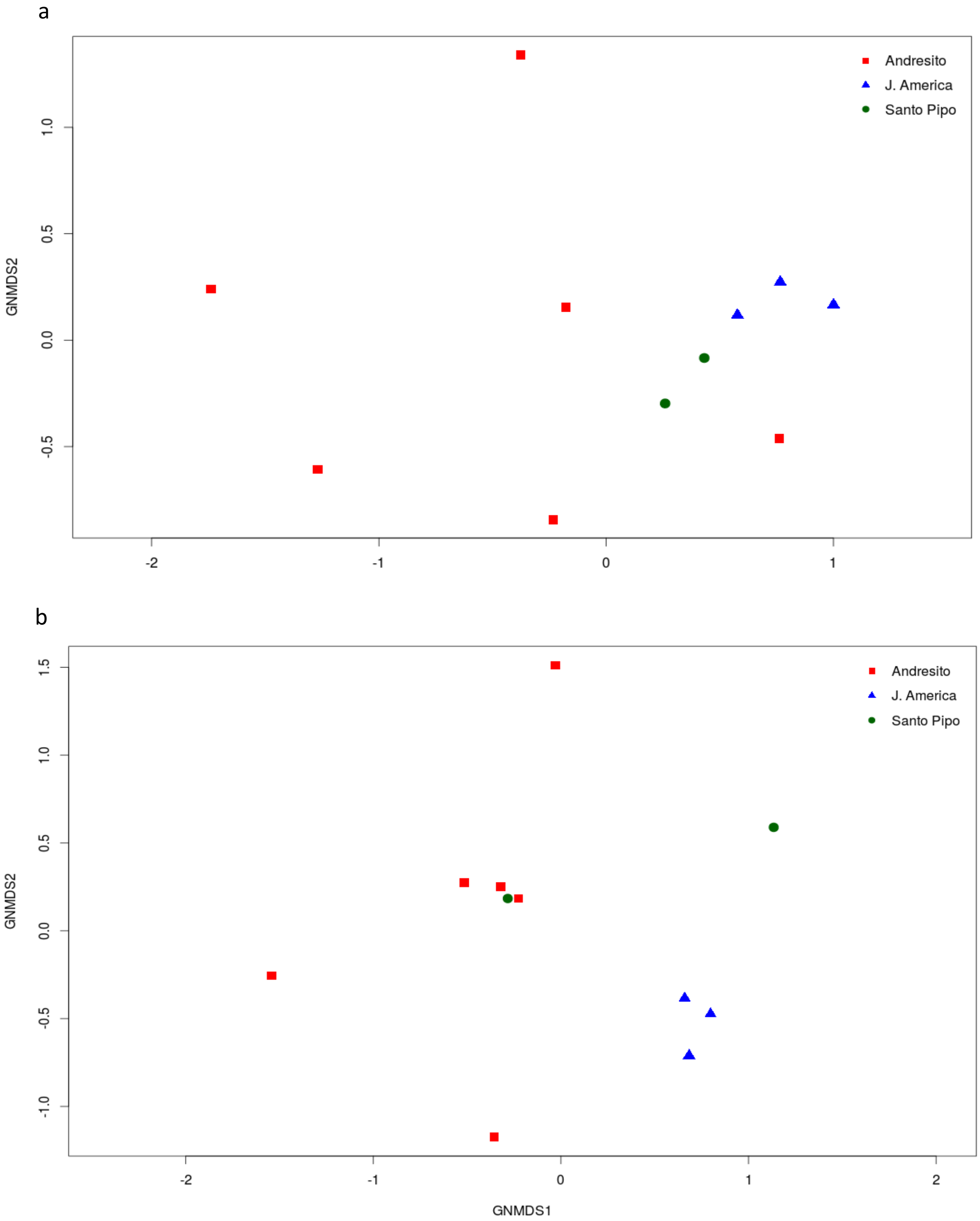


Figure 3. Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distances among (a) the bacterial communities (two dimensions, stress: 10.20%) and, (b) fungal communities (two dimensions, stress: 9.94%) associated to Yerba Mate roots in the state of Misiones, Argentina (final stress =0.13). Symbols represent the location sites of the plantations.

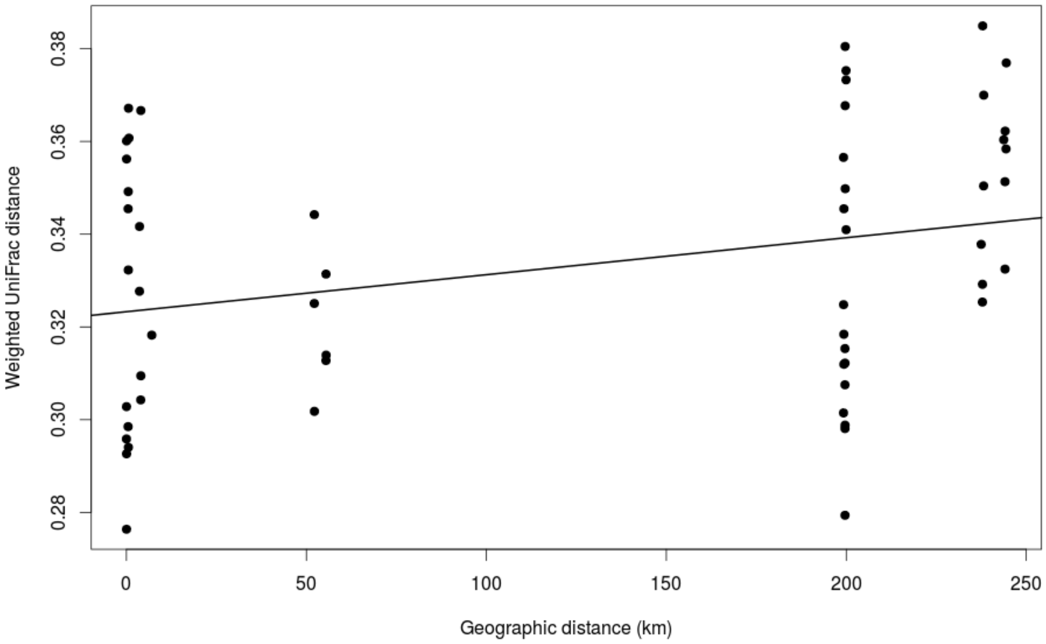


Figure 4. Weighted UniFrac distance indicating a distance-decay effect on the bacteria microbiome. The bacterial community dissimilarity is significantly and positively correlated with geographic distance (R^2=0.07, $p<0.05$), confirming that distant bacterial communities tend to be more dissimilar than geographically close bacterial communities associated with Yerba Mate roots.

Our results suggest that a local and regional scale effect might explain the similarities on the bacterial microbiomes from the south-located plantations. In recent studies, the geographical location of the plantations was the main source of variation among the bacterial root-associated microbiomes of maize and rice at a regional and local scale, respectively (Pfeiffer et al. 2013, Edwards et al. 2015). However, for the plantations located in Andresito (northern region) other unmeasured factors might be also contributing in a major degree. We hypothesized that the heterogeneity on the local edaphic variables or other unmeasured factors such as plant genotype (Aira et al. 2012) or local climatic conditions (Meier et al. 2008) might account as potential sources of variation. Regarding the fungal microbiome we observed that location and soil pH are significantly contributing to the variations. In our survey, we hypothesize that different soil pH among contiguous plantations in Andresito might contribute to increase the dissimilarity between the microbiomes, whereas a similar soil pH among contiguous plantations in Jardin America might contribute to decrease the dissimilarity between them. Previously, soil pH has been reported to have an effect on the fungal community composition at a field scale (Rousk et al. 2010), which was consistent with a global scale survey (Tedersso et al. 2014). Conversely to our results, root-associated microbiomes of *Populus deltoides* showed neither to be determined by geographical location nor by the edaphic variables such as soil pH, suggesting that a host effect can be considered as a dominant factor shaping the community composition (Gottel et al. 2011).

Analysis of core microbiomes

Firstly we analyzed if a common OTU occurred in all samples with a relative abundance of at least 1% to reveal if there is a selection of particular taxa by the plant host, independently of the geographical location and the agricultural management practice of the plantations. A unique bacterial OTU, assigned to the genus *Acidibacter-like,* occurred in all samples representing approximately 7% of the total relative abundance. The selection of this group by the acidic soil conditions has been discussed previously. In addition, we analyzed which are the distinctive bacterial OTUs associated to plantations of high, medium and low productivity yields. Six distinctive OTUs identified as *Burkholderia* (2 OTUs) *Enterobacter, Phenylobacterium, Acidibacter-like* and *Rhodanobacter* occurred with more than 1% in all low productivity plantations independent of their geographical location. We hypothesize that some of the species from the genera *Burkholderia* and *Enterobacter* might play a relevant role in favoring plant nutrition since many plant growth-promoting bacterial species have been reported within these genera (Taghavi et al. 2009, Suárez-Moreno et al. 2012). In a previous study phosphate solubilizing bacteria from the genera *Burkholderia* and *Enterobacter* have been isolated from Yerba Mate rhizosphere (Collavino et al. 2010), suggesting that they might be involved in facilitating phosphorus acquisition to Yerba Mate trees in field conditions. In addition to this, the plant growth promoting rhizobacteria (PGPR) *Kosakonia radicincitans* YD4 (family *Enterobacteriaceae*) isolated from a soil-degraded plantation was the most promising bio-inoculant to improve biomass yields of Yerba Mate seedlings (Bergottini et al. 2015). We hypothesize that the rhizosphere of Yerba Mate trees in soil-degraded or low-productive plantations may harbor potential PGPR species of the genera *Burkholderia* and *Enterobacter,* and therefore, efforts should be concentrated in isolating these bacterial species as potential PGPRs. The common OTUs assigned to *Phenylobacterium* and *Rhodanobacter* might reflect the use of herbicides (discussed previously) and an enrichment of nitrates in soil due to nitrogen fertilization, respectively.

On the other hand, one common fungal OTU assigned to *Fusarium oxysporum* was identified in all samples representing approximately 5% of the total relative abundance. In agricultural soils *F. oxysporum* is responsible of causing wilt diseases in many crops (Armstrong and Armstrong 1981), however there are non-pathogenic strains used as bio-control agents (Postma and Rattink 1992). In contrast, other fungal OTU common within the medium and higher productive plantations could not be associated to plant nutrition or protection.

5.5. Conclusion

This study describes for the first time the bacterial and fungal communities associated to Yerba Mate roots in plantations with different historical management practices. Using high-throughput 454-pyrosequencing we revealed that the rhizoplane and endosphere niches of this crop harbor microbial taxa potentially involved in plant growth promotion and pathogenesis. Our results suggest that future efforts should be concentrated in the isolation and selection of putative PGPRs strains from the genera *Burkholderia* and *Enterobacter.* A first insight into the endomycorrhizal communities associated with this crop revealed that species of *Acaulospora* are particularly enriched in plantations. However, further analysis using specific primers to tag the *Glomeromycota* phylum will provide higher resolution for the identification at the species-level. The fact that the nature of many causal agents of Yerba Mate diseases are still unknown, the predominance of unclassified genera of the orders *Hysteriales* and *Marangiales* might suggest to consider them as potential phytopathogens. Regarding the factors structuring the composition of the microbial communities, further analysis including more plantations sites with contrasting edaphic properties located across the state Misiones should contribute to reveal those main factors determining the alpha and beta diversity. In addition, future analysis should include bulk samples to confirm a plant host recruitment effect on the soil microbial reservoir.

5.6. Acknowledgements

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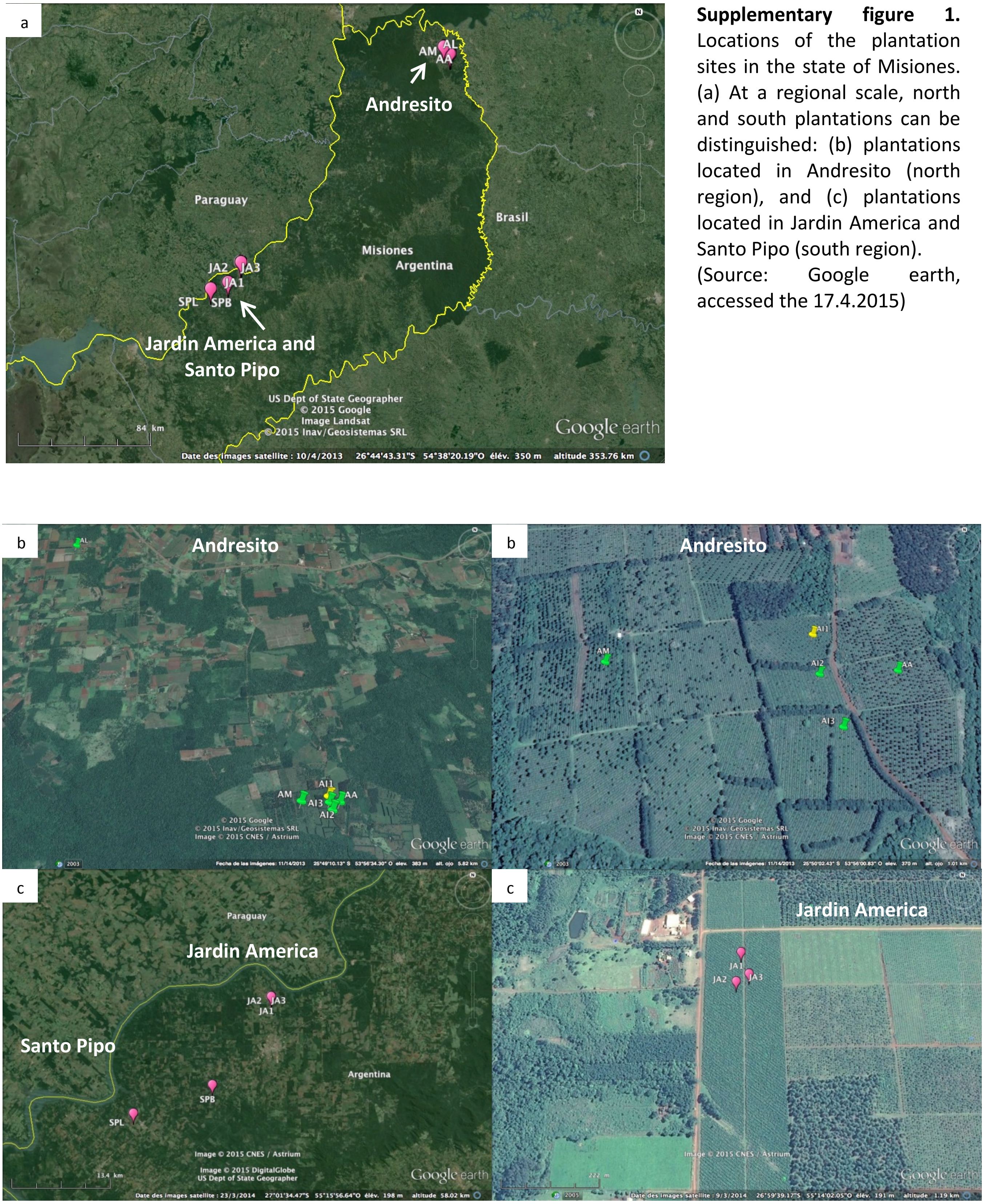

Supplementary figure 1. Locations of the plantation sites in the state of Misiones. (a) At a regional scale, north and south plantations can be distinguished: (b) plantations located in Andresito (north region), and (c) plantations located in Jardin America and Santo Pipo (south region).
(Source: Google earth, accessed the 17.4.2015)

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Chapter 6: Synthesis

6.1. General discussion

The rapid degradation of soils due to long-term inadequate agricultural practices in Misiones, the major productive region of Yerba Mate in Argentina, have led to an urgent need for research and development of more sustainable agricultural practices in order to delay, and if possible revert, the consequences of soil impoverishment (INYM-INTA 2008). This thesis contributes with an environmental-friendly alternative approach that could be exploited to improve the growth of Yerba Mate in low fertility soils.

The principal findings of our research were i) bio-inoculation with native plant growth promoting rhizobacteria (PGPR) represents an eco-friendly alternative to improve the growth of Yerba Mate seedlings in less fertile soils; ii) the most promising PGPR strain *Kosakonia radicincitans* YD4 presents the genetic elements (genes for chemotaxis, adherence structures and antimicrobial activities) to colonize successfully the rhizosphere of Yerba Mate; iii) the presence of the gene clusters for Type III and Type VI secretion systems were associated to mechanisms of antagonism against other microbes rather than to a potential pathogenicity; iv) Yerba Mate roots harbor bacterial and fungal taxa that could potentially contribute to plant nutrition and protection, guiding future isolation and selection of plant growth promoting bacteria and mycorrhizal fungi for biotechnological applications; and v) the bacterial root-associated microbiome of Yerba Mate is significantly influenced by the location of the plantation sites, whereas the fungal microbiome by the location and soil pH of the plantations.

Firstly, we were able to demonstrate that PGPR strains isolated from Yerba Mate roots increase significantly biomass yields of seedlings in nursery. There are two key findings of this part of our study that are worth highlighting: i) Yerba Mate isolates increased biomass yields in contrast to *Azospirillum brasilense* 245*,* despite the fact the latter is a well-characterized PGPR used for bioinoculation in different crops ii) bio-inoculation increased biomass yields in soil but not in organic compost (the typical substrate used in nurseries). In compost, we only observed a positive effect of the bio-inoculation on the content of some macronutrients. The higher effectiveness of Yerba Mate isolates might reflect a better adaptation to local environmental conditions (i.e. low soil pH, temperature, moisture) and crop specificity. The fact that the PGPR strains did not enhance the growth of *Phaseolus vulgaris* might support this plant species-specificity. A previous study has shown that higher yields can be obtained with the crop from which bio-inoculants were isolated (Mäder et al. 2011). Regarding the performances of the PGPR strains, *Kosakonia radicincitans* YD4 was the most promising bacterial inoculant among the three isolates tested. The potential of YD4 was confirmed when the biomass yields of seedlings inoculated with this strain were compared to those obtained in compost. We expected to obtain higher yields in compost due to its good fertility independently of the inoculation treatments. However this was not the case. These results were in agreement with a previous study in which bio-inoculation exerted a more pronounced effect of plant growth in less fertile soils (Mäder et al. 2011).

We hypothesize that the mechanism of growth promotion of *K. radicincitans* YD4 may be associated with a synergic combination of the different PGPR activities of this strain (IAA-like compounds, siderophores production, and N_2 fixation). However, this hypothesize should be approached experimentally. For instance, knocking out genes involved in these PGPR traits will allow to compare the performance as bio-inoculants of mutants versus wild type strains.

On the other hand, we want to mention some important methodological issues that should be taken into consideration if further bio-inoculation assays are going to be performed. After considerable and fruitless efforts in trying to cultivate Yerba Mate *in vitro,* we consider that the strategy to select PGPR strains for this crop cannot follow the typical analytical stages performed in other plant species. Our experience suggests that growth-chamber tests should not be approached; therefore nursery assays should represent the starting point to test a putative bio-inoculant for this crop. Regarding the traceability of the PGPR strains in the rhizosphere, techniques with higher sensitivity such as quantitative PCR should be used instead of fingerprinting approaches that were ineffective to trace and detect the prevalence of these inoculants strains in soils.

Besides exerting a plant growth promotion or protection effect, a PGPR strain should be able to colonize successfully the rhizosphere and should not represent a potential sanitary risk for the environment and human health. In the third part of the thesis the genetic basis necessary to infer the rhizosphere competence ability of the PGPR strain YD4 were analyzed. Within the genome of YD4 genes involved in chemotaxis, motility, adherence structures and catabolism of plant-derived compounds were identified, reflecting the potential ability of this strain to colonize successfully the rhizosphere. Regarding the safety of using YD4 as bio-inoculant the presence of genes associated with pathogenicity were analyzed in the genome of this strain. We identified gene clusters for the Type III (T3SS) and Type VI (T6SS) secretion systems, since these latters have been associated with the pathogenicity of some bacterial strains (Tampakaki 2014, Shrivastava and Mande 2008). In order to infer their potential role in *K. radicincitans* YD4 we performed comparative analysis with closely related strains involved in plant growth promotion and pathogenicity. We observed that the phytopathogenic strain *K. radicincitans* UMEnt01/12 does not present T3SS but presents T6SS, whereas the plant growth promoting endophyte *K. radicincitans* DSM 16656^T does not present T3SS but presents T6SS. These results suggest that the presence of these secretions systems in the genome of YD4 could not be a pre-requisite for developing a potential pathogenic lifestyle. On the other hand, T3SS was founded to be a dispensable trait for nodulation of legumes in symbiotic rhizobia (Tampakaki 2014), whereas the possession of the T6SS in non-pathogenic bacterial strains has shown to be associated with interbacterial interactions enhancing their survival and competitiveness in their habitats (Schwarz et al. 2010). According to this, we hypothesize that both secretions systems in YD4 strain might be a competitive advantage for the interactions with other microbes and simple eukaryotic cells in the rhizosphere. In a recent study, Bulgarelli et al. (2015) has shown that the rhizosphere of barley is enriched in specific bacterial genes related to traits for survival and adaptation to the root niche, for instance the T3SS and T6SS, siderophore production, and sugar transport. Combining our comparative genomic analyses, we hypothesize that *K. radicincitans* YD4 presents the genetic potential to colonize and survive competitively in the rhizosphere niche.

In order to confirm the hypothesis that *K. radicincitans* YD4 is a competitive PGPR strain we performed a second inoculation assay in nursery to detect and quantify the introduced bacterial inoculant in the rhizosphere of Yerba Mate. We have observed that *K. radicincitans* YD4 increased significantly the dry weight of seedlings in soil as observed previously, even though no increase in the content of macronutrients occurred in shoots. We confirmed that this bacterial inoculant is contributing to increase the biomass yields of seedlings and we are optimistic with the idea that this positive effect on plant growth can be reproduced in field experiments. To address the main objective of this study, a quantitative real-time PCR approach proposed in a previous study (Ruppel et al. 2006) and modified to tag specifically the PGPR strain YD4 was conducted. However, when the primer specificity control test was performed, amplification with a significant number of copies was obtained in our negative control strain, reflecting the non-specificity of this approach. Therefore, we decided to design a nobel strain-specific primer set to monitor by qPCR our bacterial inoculant. Performing comparative analysis with the available genomes of other species of the genera *Kosakonia* and *Enterobacter,* 128 unique coding sequences (CDSs) were identified in YD4 genome. Then, these singletons were analyzed using the NCBI Blast search against the GenBank database in order to select those unique CDSs for the strain YD4. One candidate CDSs (LG58_6128) was selected for designing a strain-specific primer for YD4. After confirming its YD4-specificity by PCR amplification with other bacterial strains we monitored the dynamics of this strain in the rhizosphere of inoculated Yerba Mate seedlings during five weeks. According to our results, the introduced strain YD4 was able to colonize and persist within the rhizosphere during the five weeks the experiment lasted. After its application in soil (5 ml of a bacterial suspension of 10^8 cells mL^{-1} in pots of 200 cm^3), YD4 population remained almost constant in the rhizosphere and reached 5.77 x 10^4 number of copies per gram of root at the fifth week. This study allowed to attribute the plant growth-promoting effect observed in seedlings with the presence of the introduced bacterial inoculant YD4 within the rhizosphere. In addition, it provides a strain-specific qPCR approach to monitor this bacterial inoculant in soil.

This thesis represents the beginning of a future project (with continuity in Argentina) that aims at the development of a native microbial consortium, including bacterial and fungal plant growth promoters to improve Yerba Mate growth in low-fertility soils. To achieve this goal in the future, it was crucial to investigate the bacterial and fungal communities associated to this crop in the field to guide the isolation and selection of enriched microbial taxa with potential biotechnological applications. Using high throughput 454-

pyrosequencing our study revealed that the root-associated microbiome of Yerba Mate is enriched in bacterial taxa including *Burkholderia* and *Enterobacter*, from which potential PGPR strains can be isolated. PGPR strains contributing to plant nutrition and protection have been reported for both bacterial genera (Suárez-Moreno et al. 2012, Liu et al. 2013). In a previous study, phosphate-solubilizing bacteria belonging to these genera were isolated from Yerba Mate roots, however their potential as bio-inoculant was tested in another plant species (Collavino et al. 2010). Regarding the fungal taxa, *Glomeromycota* was one of the most abundant phylum detected (5% of the total relative abundance). Interestingly, a shift in the relative abundance of *Glomeromycota* was observed in one of the low-productive plantations that presented lower P content. This might reflect the fact that in low-input fields trees are exerting a selective pressure for mycorrhization, as observed in others plant species (Mäder et al. 2000). Hence, the importance of studying in more details those endomycorrhizal taxa that are sustaining Yerba Mate nutrition in low-productive fields. Unfortunately, our pyrosequencing reads did not allow identification at the species level and the majority of the *Glomeromycota* OTUs were assigned to unclassified genera of the family *Acaulosporeacea.* Further analysis using specific primers for the *Glomeromycota* may provide higher resolution at the species level. Besides the mycorrhizal fungi, we want to highlight that the two most abundant fungal genera assigned as unclassified fungi of the orders *Hysteriales* and *Marangiales*. Since some genera of *Hysteriaceae* (*Hysteriales*) and *Elsinoacea* (*Myriangiales*) are phytopathogens (Alexopoulus et al. 1996, Jayawardena et al. 2014), the relative abundance of these OTUs could suggest considering these taxa as potential pathogens for this crop. A more detailed analysis of these genera could shed light on pathogenesis in Yerba Mate since the majority of the causal agents of Yerba Mate diseases are still not known.

On the other hand, the root-microbiome survey gave a first insight into the factors contributing to determine microbial community composition, genetic and phylogenetic diversity. Although we only included eleven plantations in this study, we analyzed if the agricultural historical management (high, medium and low productive plantations) and/or the geographical location (northern versus southern) of the plantations contributed to explain the alpha and beta diversity of the root-associate microbiome. The alpha diversity could not be related to these factors. However, one Yerba Mate plantation established in a rainforest-logged field and with no-tillage practices presented a notably higher bacterial richness. Regarding the beta diversity, the geographical location of the plantations accounted as a source of variation in the bacterial community composition, genetic diversity and phylogenetic diversity. A local scale effect explained the similarities observed among south-located plantations (Jardin America and Santo Pipo), while for the north located (Andresito) we hypothesize that heterogeneity in local environmental factors might explain the dissimilarity among microbiomes. Plant genotype (Aira et al. 2012) or local climatic conditions (Meier et al. 2008) might account as potential sources of variation. For the fungal microbiome the geographical location and soil pH of the plantations contributed to the variations. Plantations stands of Jardin America presented similar fungal microbiomes, an observation that can be explained due to their contiguous location and similar soil pH. However this trend was neither observed within the contiguous stands in Andresito nor in those in Santo Pipo. Since in a previous study a gradient of pH across 180 meters within a field affected (slightly but significant) the fungal community composition (Rousk et al. 2010), we hypothesize that the different soil pH of the contiguous plantations stands in Andresito influenced the variations in the fungal communities. In order to obtain a broader understanding of the factors contributing to determine the root-associated microbiome of Yerba Mate, further analysis should include a higher number of plantation sites with contrasting soil properties located across the state Misiones.

6.2. Conclusion

The research conducted in this thesis has demonstrated that Yerba Mate growth can be improved through bio-inoculation with native plant growth promoting rhizobacteria (PGPR) in less fertile soils. The isolation, characterization, and selection of a limited number of rhizobacteria strains have demonstrated that Yerba Mate roots harbor effective PGPR strains that can be exploited for further biotechnological applications. In addition, the enrichment of members from the genera *Burkholderia* and *Enterobacter* in Yerba Mate roots in different plantations, suggests that future efforts should be concentrated in isolating these bacteria. In this study, the most promising PGPR strain: *Kosakonia radicincitans* YD4, has shown that higher yields can be obtained through bio-inoculation in less fertile soils in comparison to higher fertile soils in nursery conditions. In addition, this PGPR strain has shown to persist in the rhizosphere of Yerba Mate seedlings after bio-inoculation.

Prior to our studies, the role of native microbes as potential bio-inoculants for this crop has not been assessed as a sustainable alternative to improve plant growth in less fertile soils. At the end of this thesis we are able to suggest that bio-inoculation with native PGPRs should be considered as an eco-friendly strategy to enhance the growth of Yerba Mate seedlings in nursery conditions.

6.3. Outlook

As mentioned in the general discussion, this thesis represents the beginning of a future project that aims at the development of a native microbial consortium, including bacterial and fungal plant growth promoters, to improve Yerba Mate growth in low-fertility soils. The role of *K. radicincitans* YD4 as bio-inoculant should be evaluated in field trials in order to confirm the promising growth-promoting effect observed in nursery. This could be possible with the collaboration of local specialist in Yerba Mate cultivation and we are optimistic with the idea that this can be approached in the near future. According to the results obtained in this thesis, we are able to suggest that future studies should include the evaluation of mycorrhizal symbiosis on the growth of Yerba Mate since co-inoculation PGPR-Mycorrhizae could boost even more the plant growth-promoting effect on this crop. Since our study has shown a shift in the phylum *Glomeromycota* in a low-productive plantation, the analysis of the mycorrhization rate of roots in these types of plantations could give an indication of the relevance of this symbiosis in low-input plantations.

On the other hand, the whole genome sequence of the PGRP strain YD4 could allow to perform fundamental research to elucidate the mechanisms of plant growth promotion or the rhizosphere competence traits of this strain. For instance, by producing strains knocked out in genes of the secretions systems Type III and Type VI will be possible to compare the rhizosphere colonization ability of the mutants and wild type strains. This will allow to evaluate our hypothesis that these genes clusters can confer fitness advantages to the strain YD4 in the competitive rhizosphere niche. Another interesting approach will be to analyze the performance of mutant strains knocked out in genes responsible for some of the PGPR traits of YD4 to evaluate if one of them is critical for exerting its plant growth-promoting mechanisms.

In our study we could not observe a significant effect of the agricultural management on the fungal and bacterial communities. However, it will be of high interest to explore and identify potential biological indicators of soil quality. This could provide recommendations about the agricultural practices that promote and conserve the biological diversity of Yerba Mate agroecosystems.

6.4. References

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Publications

2015	Bio-inoculation of yerba mate seedlings (*Ilex paraguariensis* St. Hill.) with native plant growth promoting rhizobacteria: a sustainable alternative to improve crop yield. Bergottini V, Otegui M, Sosa D, PD Zapata, M Mulot, M Rebord, J Zopfi, F Wiss, B Benrey, P Junier. *Biology and Fertility of Soils. In press*
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2011	Comparative symbiotic performance of native rhizobia of the Flooding Pampa and strains currently used for inoculating *Lotus tenuis* in this region. Sannazzaro AI*, Bergotttin V*, Paz RC, Castagno LN, Menéndez AB, Ruiz OA, Pieckenstain FL, Estrella MJ. *Antonie van Leeuwenhoek,* 99:371–379. *Equally contributed.
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Professional experience

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Conferences

2014	Bio-inoculation of yerba mate with plant growth promoting rhizobacteria: a sustainable alternative for increasing crop yield. 15th International Symposium of Microbial Ecology. Seoul, South Korea, 24-29 September. Bergottini V *et al*. (Poster)
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