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Bacterial community composition in rhizosphere microsites of ryegrass grown in grassland soils

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Bacterial community composition in rhizosphere microsites of ryegrass grown in grassland soils

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Summary and thesis outline

The rhizosphere, defined as soil zone affected by plant roots, is a complex environment where diverse microorganisms have an important role in nutrient cycling and plant growth. Thus, the first chapter gives a brief introduction about heterogeneous distribution of bacterial communities in different root locations or microsites according to diverse biotic and abiotic factors, such as root exudates, fertilization, type of soil, etc. In addition, this chapter also includes the overall objective of this doctoral thesis.

Considering that our knowledge of the ecology and role of bacteria in the rhizosphere is very limited, in the second chapter we present a review which provides an overview of the current knowledge on the study of bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of classical molecular techniques, next generation sequencing platforms and post–genomics techniques.

Moreover, in order to examine the bacterial community ecology in the rhizosphere of ryegrass grown in Chilean Andisol, the third chapter provides a description of diversity of bacterial communities in rhizosphere microsites (root tip and mature zone) of ryegrass grown in soil from grassland with P availability (Freire and Piedras Negras serie) and under greenhouse conditions. In this context, our results shown in detail the rhizobacterial communities associated ryegrass and revealed the high proportion of low-abundance of bacterial groups, including novel members do not described for soil and rhizosphere at the present.

Considering that rhizobacterial communities may be crucial in phosphorus (P) nutrition of plants, our efforts were focused in to know how P fertilization modulates rhizobacterial communities. Thus, in the fourth chapter we shown the effect of P addition (phosphate and phytate) on total bacterial communities and alkaline

phosphomonoesterases (APase) gene-harboring bacterial populations in the rhizosphere microsites (root tip and mature zone) of ryegrass. Our results suggested that P addition induces changes on the richness and abundance of rhizobacterial communities.

Finally, the fifth and sixth chapters give an overall summary of the conclusions derived from the obtained results and also discusses future research needs to facilitate better understanding of rhizosphere conditions and P fertilizer practices. In order to improve the plant P nutrition or the application of biofertilizers (e.g., plant growth-promoting rhizobacteria).

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Chapter 1

General Introduction

1. General Introduction

1.1 Introduction

The rhizosphere bacterial community is the main reservoir of active microorganisms present in soil, where diverse microorganisms contribute to nutrition, growth, and disease suppression of plants (Bastidas et al. 2009). Given these important functions, there is considerable interest in understanding the linkage among microbial community structures, soil management practices, and plant growth in different types of soils. Rhizosphere communities also are highly spatially and temporally dynamic due changes in the abundance and diversity of bacterial communities produced by chemical nature of root exudates that serve as Carbon (C) and energy sources for microorganisms (Bertin et al. 2003), as well as the influence of abiotic factors (pH, organic matter, soil type, etc.) that affect the selection of different microbial species (Gray and Smith 2005; Marschner et al. 2011; Rovira 1965; Yang and Crowley 2000). Recent studies on rhizobacterial communities have also shown variation along the root axes in relation to the concentrations and types of labile C that are deposited into the rhizosphere. According to current models, labile C is the greatest in the zone of elongation and it is postulated to drive a successional process, in which the distal zone selects growth of opportunistic copiotrophic bacteria. Copiotrophic bacteria are competitive in uncrowded environments, which are followed by a succession of other microbial species that are adapted to an increasingly crowded and C limited environment in older root zones (Marschner et al. 2011; Yang and Crowley 2000).

On the other hand, Phosphorus (P) is an essential nutrient for plant and microorganism metabolisms. In the rhizosphere, diverse bacteria groups have shown capacity to solubilize inorganic P (Pi) and mineralize organic P (Po) (Richardson, 2001). The microorganisms have the ability of solubilize Pi through acidification of soil

by organic acids exudation (Miller et al. 2010; Uroz et al. 2010). The mineralization of Po is carry out by release phosphatase enzymes, such as phosphomoesterases (Nannipieri et al. 2011). In relation to study of alkaline phosphomonoesterases (APase), they are exclusively associated with metabolism of microorganisms, such as bacteria and fungi, while acid phosphomonoesterases are released by both plants and microorganisms. The main function of APases are hydrolyzing of organic phosphorus (Po) to inorganic phosphates under P-limiting conditions. The families ALP are phoD, phoX and phoA, being phoD the most abundant in soils (Tan et al. 2013). Diverse studies have observed that the diversity and abundance of *phoD*, *phoX* genes are affected by P fertilization (Chhabra et al. 2013; Fraser et al. 2015). Chilean Andisol is characterized by high content of Po, but low P availability. Moreover, APase genes have been reported in acidic and alkaline Chilean soils, suggesting that APase could contribute to P cycling in Chilean extreme environments (Acuña et al. 2016). Nevertheless, our knowledge about P fertilization effect on nutrients cycling, availability, and its influence on ecology of rhizobacterial communities along roots still is poor.

Considering that rhizosphere is complex environment have been crucial to study the bacterial ecology by molecular techniques, which has allowed the characterization of uncultured organisms at level never before seen. Molecular techniques based on DNA has been an important tool used to know microbial community structure, identify abundance genes that could be involved in rhizosphere processes. In this context, the use of metagenomics, defined as the genomics based study of genetic material recovered directly from environmental samples without laboratory culture, appears as a powerful tool to know more accurately the microbial diversity involved in the rhizosphere dynamics and its influence in plant growth (Arjun and Harikrishnan, 2011).

Allison et al. (2008) postulate that metagenomics analysis may lead our understanding on plant-microorganism-soil system, allow us to evidence the importance of certain microbial functional groups and know the responses of microbial communities to various disturbances in agriculture soils. With the advent of high-throughput sequencing technology (such as 454-sequencing), it has become now possible to re-examine the structure, diversity and activity of microorganisms at much higher resolution in more detail. These high resolution analyses of the taxonomic composition of the rhizosphere provide baseline information on the specific microflora that occur in the rhizosphere, from which we can thereafter infer the community structures at specific root zones, and their functional relationships with plant growth, nutrient uptake and cycling.

1.2 Hypothesis and research objectives

The bacterial composition along rhizosphere can be influenced by various biotic and abiotic factors such as plant species, soil type and agronomic practices. Moreover, the composition of rhizobacterial communities differs along the length of roots due to selective enrichment of different populations according to the amount and composition of root exudates, resulting in a heterogeneous distribution of rhizobacterial communities. Therefore we propose the following hypothesis

1.2.1. Hypothesis

Rhizobacterial community composition at microsites level is modified by type soil and supplied P sources at soil, generating a higher diversity (abundance and richness) of bacterial community in root tip in comparison to mature zone. Moreover, supplied P source and its availability at rhizosphere microsites level regulates the abundance of functional genes related to P cycling.

1.2.2. Research Objectives

1.2.2.1 General Objective

To determine the bacterial communities composition in rhizosphere microsites of ryegrass (*Lolium perenne* var. Nui) grown in grassland soils under greenhouse conditions

1.2.2.2 Specific Objectives

S.O 1: To describe the diversity of bacterial communities in microsites of ryegrass rhizosphere grown in two grassland soils with different agronomic managements.

S.O 2: To assess the changes produced by added P source on bacterial communities composition in rhizosphere microsites of ryegrass

S.O 3: To assess the changes produced by added P source on APase-harboring bacterial populations and its effect on abundance of two genes encoding APase (*phoX* and *phoD*).

CHAPTER 2

Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: A mini-review

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Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: A mini-review

Abstract

The rhizosphere (soil zone influenced by roots) is a complex environment that harbors diverse bacterial populations, which have an important role in biogeochemical cycling of organic matter and mineral nutrients. Nevertheless, our knowledge of the ecology and role of these bacteria in the rhizosphere is very limited, particularly regarding how indigenous bacteria are able to communicate, colonize root environments, and compete along the rhizosphere microsites. In recent decades, the development and improvement of molecular techniques have provided more accurate knowledge of bacteria in their natural environment, refining microbial ecology and generating new questions about the roles and functions of bacteria in the rhizosphere. Recently, advances in soil post–genomic techniques (metagenomics, metaproteomics and metatranscriptomics) are being applied to improve our understanding of the microbial communities at a higher resolution. Moreover, advantages and limitations of classical and post–genomic techniques must be considered when studying bacteria in the rhizosphere. This review provides an overview of the current knowledge on the study of bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of classical molecular techniques, next generation sequencing platforms and post–genomics techniques.

Keywords: Microbial ecology, molecular techniques, rhizosphere, next–generation sequencing

2. Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: a mini-review

2.1. Introduction

The rhizosphere, is defined as the soil zone under the influence of plant roots, a site of high microbial activity, characterized by a great array of complex and dynamic physical, chemical and biological interactions. In the rhizosphere, microorganisms have an important role in the organic matter transformations and biogeochemical cycles of plant nutrients. In this context, a substantial number of bacterial species interact with their host plants and may exert beneficial effects on plant growth, plant nutrition and disease suppression (Avis et al. 2008, Pii et al. 2015). Thus, numerous studies have been devoted to isolate and describe the activities of diverse plant growth−promoting rhizobacteria (PGPR). Among the main functions attributed to PGPR are: biological N_2 fixation, phytopathogen biocontrol, phosphate solubilization, production of phytohormones and enzymes, among others. Nowadays, PGPR represent an attractive alternative for chemical fertilizers as biofertilizers, phytostimulators, rhizoremediators and phytopathogen biocontrol agents (Lugtenberg and Kamilova, 2009). Despite the perception that PGPR are considered as not dangerous to the environment, their mass application in agriculture have been limited due to their low efficiency in field–level applications (Babalola, 2010). There is still a lack of information on PGPR ecology (diversity, competence, distribution, communication, etc.) in the rhizosphere (Compant et al. 2005; Lambers et al. 2009; Singh et al. 2011); however, a considerable interest has been generated in their study and potential use in crop production around the world (Morrisey et al. 2004).

Currently, it is recognized that the rhizosphere microbiome harbors thousands of different bacterial, archaeal, viruses, fungal and other eukaryotic taxa. A gram of rhizosphere soil might contain around 10^9 microbial units and 10^6 distinct taxa (Torsvik et al. 2002; Curtis and Sloan, 2005). The use of molecular techniques have allowed the characterization of unculturable organisms at a level never seen before, since only 1% of soil microorganism have actually been cultured (VerBerkmoes et al. 2009; van Elsas and Boersma, 2011). Molecular techniques based on DNA analyses provide important tools to evaluate bacterial community composition, identifying the abundance of genes that could be involved in rhizosphere processes. With the advent of next generation sequencing technologies (such as Roche 454 and Illumina platforms), it has become possible to characterize the composition and activity of bacteria at much higher resolution than before. Metagenomics (Handelsman et al. 1998; Myrol and Nannipieri, 2014) approaches can allow characterization of the unknown genomes of unculturable bacteria with the probability of finding novel microbial products (antibiotics, enzymes, anti–cancinogenics, etc.) (Nannipieri et al. 2008). These high resolution analyses of the taxonomic composition of rhizosphere soil provide baseline information on the specific microbiome members living in rhizosphere environments. In addition, we can thereafter infer the community composition at specific root zones, and their functional relationships with plant growth, nutrient uptake, and plant health. Moreover, both RNA and proteins (proteomics) can provide some information on gene activity, such as metatranscriptomics (total RNA from all bacterial communities of rhizosphere soils) inferring soil–microbial functionality (Bastida et al. 2009).

This review provides an overview of the current knowledge on bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of

classical molecular techniques, next generation sequencing platforms and post–genomics techniques.

2.2. Ecology of bacteria in the rhizosphere

2.2.1 Abundance and diversity

The rhizosphere harbors a wide variety of bacteria species, and the compositions of bacterial communities differ according to root zone, plant species, plant phenological phase, stress and disease events (Rovira, 1965; Hinsinger et al. 2009; Marschner et al. 2011). Dominant bacterial phyla are members of the classes Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Acidobacteria in the rhizosphere of sugarcane, pea native hardwood forest and conifer plantations (Mendes et al. 2011; Weinert et al. 2011; Yang et al. 2012). Among them, Proteobacteria are considered to be the most abundant bacterial group in the rhizosphere due to their ability to respond to labile C sources, showing fast growth and adaptation to the diverse plant rhizospheres (Bulgarelli et al. 2013; Chaparro et al. 2014; Peiffer et al. 2013). Proteobacteria is followed by Acidobacteria, which have been attributed an important role in the C cycle in soils due to their ability to degrade cellulose and lignin (Ward et al. 2009). In relation to Actinobacteria, they have been associated with disease suppressive soils and their ability to increase root nodulation and promote the growth of plants (Mendes et al. 2011; Tokala et al. 2002).

An important bacterial group in the rhizosphere is defined as PGPR, which are able to promote the growth, nutrient uptake and pathogen biocontrol in plants (Avis et al. 2008; Mendes et al. 2011). According to the current *GenBank* database from National Center for Biotechnology Information (NCBI), the most abundant groups of bacteria

which potentially can promote plant growth are Actinobacteria, Firmicutes, Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) and Bacteroidetes (Figure 2.1), and particularly the genera Rhizobium, Azospirillum, Burkholderia, Pseudomonas and Enterobacter. Other bacterial groups reported in the rhizosphere are Verrumicrobia, Sphingobacteria, Flavobacteria, Deinococcus, Fusobacteria, and Epsilonproteobacteria. These bacterial groups are not commonly observed as dominant groups and/or PGPR in the rhizosphere, although this may be due to our limited knowledge of their taxonomy and functional gene sequences. It is also possible the presence of low abundance bacterial group with specific metabolic capabilities and/or adapted to the characteristic of specific plant species rhizosphere, which make that process of soil DNA extraction to be insufficient to be detected. In addition, studies have described the influence of agriculture practices and soil pH on bacterial community composition (Cheng et al. 2010; Jorquera et al. 2014; Pisa et al. 2011; Malik et al. 2013; Chen et al. 2013; Nguyen et al. 2013), as well as the occurrence of bacteria species having dependence on other rhizosphere microorganisms for their own growth (Hirsch et al. 2010).

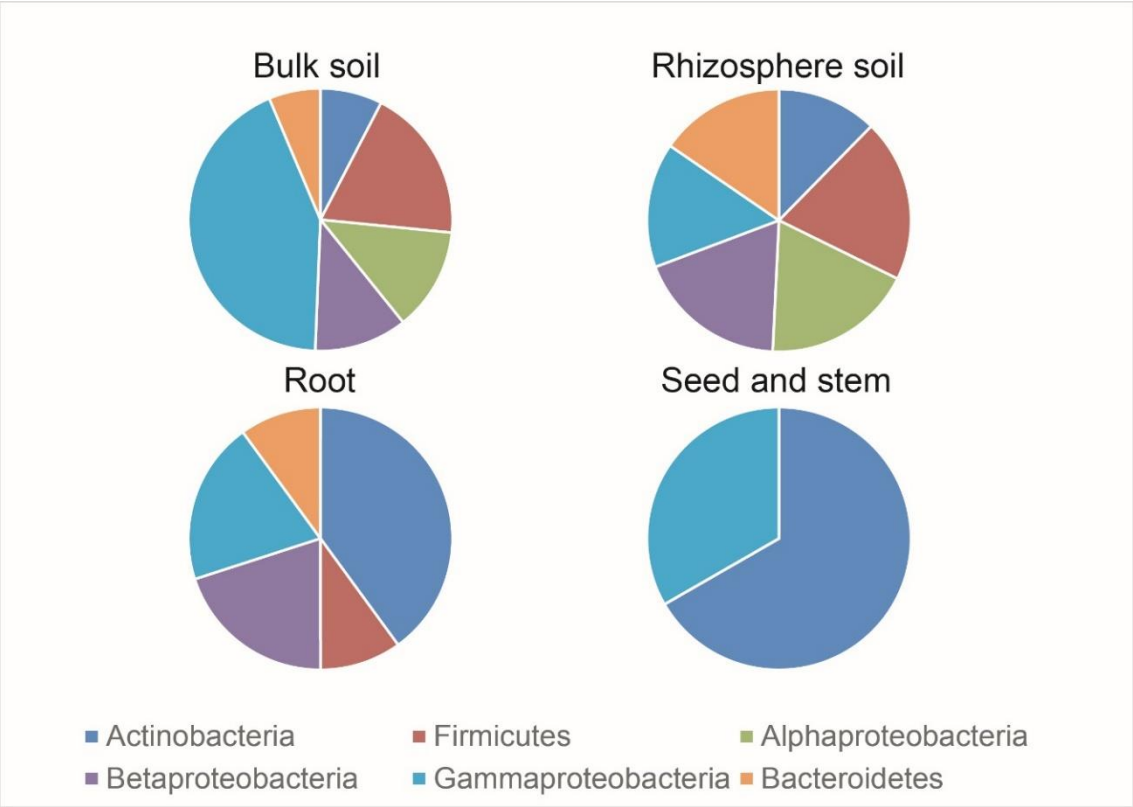

Figure 2.1 Taxonomic affiliation of 157 entries of plant growth−promoting rhizobacteria (PGPR) in GenBank database in relation to soils (bulk and rhizosphere) and plant tissues (root, seed and stem)

2.2.2 Colonization and competence

It is known that root exudates released by plants into the rhizosphere are used by bacteria as nutrient sources regulating their activities and growth. Root exudates include the releasing of ions, oxygen, water, and organic compounds, such as sugars, organic acids, amino acids, enzymes, growth factors and others (Marschner et al. 2004; Bais et al. 2004; Bertin et al. 2003; Uren, 2000). Lugtenberg et al. (1999; 2001) demonstrated that sugars and organic acids are important for tomato root colonization by bacteria and fungi. In contrast, Karlsson et al. (2012) showed that archaeal abundance was decreased by root exudates, probably as result of their lower growth rates and competitiveness compared with bacteria and fungi. The colonization of rhizosphere by bacteria also depend on their motility (Martínez–Granero et al. 2006; Raaijmakers et al. 2009; Capdevila et al. 2004), availability of inorganic compounds (P and Fe) (Raaijmaker et al. 1995;Yang and Crowley, 2000; Marschner et al. 2004; 2011) and production of antibiotics (Raaijmaker et al. 1995; Doornbos et al. 2012). In this context, *Pseudomonas fluorescens* strain F113 has been used as model microorganisms for studying the root colonization, motility and competence of the indigenous microorganisms of the rhizosphere. Thus, Capdevila et al. (2004) revealed that the motility is essential for root colonization, and that non–motile mutant strains were displaced from the root tip of alfalfa when competed with the wild–type strain. Hyper–motile mutant strains showed a higher colonization in distal parts of the alfalfa root. Plants also can influence microbial competition by root exudation. For example, white lupins (*Lupinus albus* L.) acidifies the rhizosphere by releasing protons from roots, and thus reducing bacteria growth, or they may excrete isoflavonoids, chitinase and glucanases against phatogenic fungi (Weisskopf et al. 2006).

2.2.3 Distribution along the rhizosphere microsites

The abundances, diversities and activities of microorganisms differ along the rhizosphere microsites, depending on depletion/availability of nutrients caused by root exudation and competition between microorganisms. It has been described that greater numbers of bacteria occur around the root tip zone (where the highest sucrose or tryptophan exudation occurs), whereas the lower bacterial abundance occurs around the mature root zone (Jaeger et al. 1999). Cellular death with subsequent cell lysis also releases debris which can be used as energy sources for microorganism to survive in the rhizosphere (Watt et al. 2006). Bacterial compositions also depend on soil type, plant species, plant growth seasons and climate conditions. Models of plant–bacteria interactions in different root zones have been proposed (Jaeger et al. 1999; Marschner et al. 2011; Yang and Crowley, 2000). These models support that root exudates are released in a differentiated manner along the root, resulting in a heterogeneous distribution of bacterial communities along root zones (Figure 2.2). In root tips and the proximal elongation root zone, the exudation rates are high and colonizers use easily metabolizable sugars and organic acids. In contrast, exudation rates and microbial densities are lower in root hair zones. In the mature root zones, the bacterial growth is lower due to low metabolizable C input (Marschner et al. 2011). In addition, we also may suppose that some bacterial populations are unique and have adapted to the rhizosphere by using specific compounds secreted from roots, and that the plant itself interferes with the associated bacterial community independent from soil type (De Campos et al. 2013).

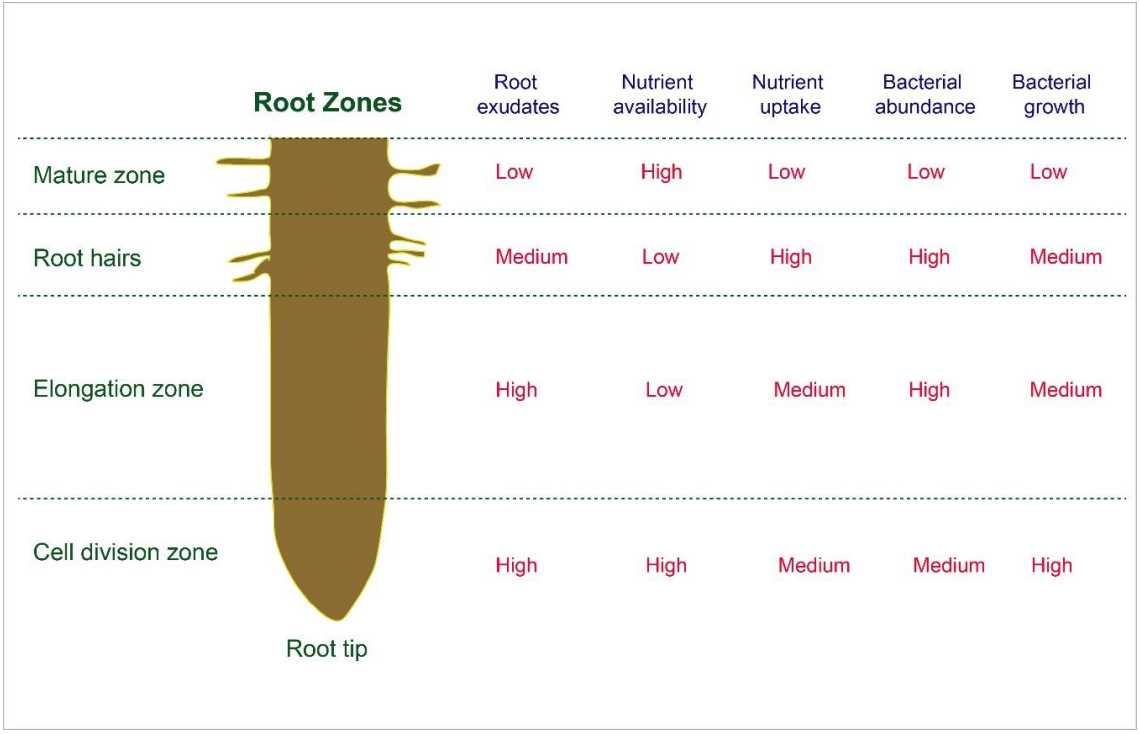

Figure 2.2 Plant and bacteria properties in different root zones according to models proposed (Jaeger et al. 1999; Marschner et al. 2011; Yang and Crowley, 2000).

2.2.4 Cell–to–cell communication

The cell–to–cell communication between bacteria in the environment is relevant due to its role in regulating vital functions, biofilm formation, virulence, symbiosis, extracellular enzyme production, antibiotic production, DNA transfer, etc. (Elasri et al. 2001; Raina et al. 2009; DeAngelis et al. 2007). The modes of signal transduction in prokaryotes linking environmental signals to cellular responses are mainly attributed to one–component and two–component systems (Ulrich et al. 2005). In general, soil bacteria can sense the presence of complex molecular exudates through a membrane–bound sensor histidine protein kinase (Fauren et al. 2009). For example, in many Gram–negative bacteria, the beneficial or pathogenic interactions with their host plant are influenced by a GacS sensor kinase and GacA transcription factor (GacS/GacA). This two–component system recognizes environmental signals involved in pathogenicity to plants (Heeb and Haas, 2001). On the other hand, an one–component system implicated in communication of the plant is NodD proteins in Sinorhizobium meliloti that perceives flavonoids and activates expression of nodulation (nod) genes, which are required for symbiotic development in alfalfa (Peck et al. 2006).

The cell–to–cell communication in bacteria may involve quorum sensing (QS), where acyl–homoserine lactone (AHL) and compounds N–acylated derivatives of L–homoserine lactone (acyl–HSL) are the main signaling molecules produced by Gram–negative bacteria, and are released to the environment in order to reflect their population density. Cha et al. (1998) showed that QS systems mediated by AHLs were 10–fold higher in the rhizosphere than bulk soil, explained by high abundance of bacterial populations in the rhizosphere. Another study revealed that *Burkholderia graminis* strains M12 and M14, plant growth–promoting rhizobacteria, can produce

AHLs that induce protection against salt stress in tomato plants (Barriuso et al. 2008). In Gram–positive bacteria, cell–to–cell communication is controlled by a two–component signal–transduction mechanisms (histidine kinase), releasing oligopeptides in response to changes in cell density (Raina et al. 2009).

Finally, it was postulated that our knowledge of cell–to–cell communication in bacteria could allow us to decipher how stable communities of PGPR can be promoted in the plant rhizosphere (Hirsch et al. 2003), involving further investigations focused on identification of AHLs produced by PGPR (Barriuso et al. 2008) and used as biosensors to understand how these molecules are involved in the diverse rhizosphere processes.

2.3. Classical molecular techniques in the study of bacteria in the rhizosphere

During the last few decades, a wide variety of molecular techniques have been developed and used as valuable tools for the study of diversity and function of bacteria in the rhizosphere (Hill et al. 2000). However, the biases of each molecular technique must be considered and evaluated during their application in the study of bacteria in the rhizosphere.

2.3.1 Fingerprinting techniques

PCR–based fingerprinting techniques, such as terminal restriction fragment length polymorphism (T–RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP), have been widely used in the study of bacterial communities in the rhizosphere (Berlec, 2012; Ascher et al. 2011). However, they determine only dominant bacterial populations within the communities, and may consider both active and inactive bacterial populations (van Elsas and Boersma, 2011). Smalla et al. (2007) compared bacterial diversities from several soils and found that T–RFLP was less efficient in assessing bacterial diversity in soil than DGGE, but T–RFLP yielded more reproducible results for comparing large numbers of samples. In general, fingerprinting techniques rely on the amplification of 16S rRNA gene fragments by PCR, but 16S rRNA gene copy number per genome vary from 1 up to 15 or more copies depending on the bacterial species. The numbers of rRNA gene copies are related to the life strategy of bacteria; taxa with low copy numbers and inhabit low nutrient environment (oligotrophic) (Větrovský and Baldrian, 2013; Kang et al. 2010).

2.3.2 Quantitative PCR (qPCR) and gene expression

The qPCR is a molecular technique widely used for detection and quantification of specific genes and their expression from DNA and RNA samples from various

environments (Deepak et al. 2007). It is a sensitive technique allowing detection of the signal produced by DNA fragment amplification in real time during each cycle of the PCR reaction. The limitations of real–time PCR are related to primer specificity, amplification efficiency, and the concentration of RNA in rhizosphere sample, which may be too low for accurate detection (Marschner et al. 2011). However, this technique can provide important insight into the distribution of specific bacterial genes in the rhizosphere and bulk soils (van Elsas and Boersma, 2011, Sørensen et al. 2009), because it is not only used to detect and quantify 16S rRNA genes but also functional genes involved in relevant processes in the rhizosphere, such as nutrient cycling and phytopathogen biocontrol.

The study of gene expression in the rhizosphere soil is still limited (Nannipieri et al. 2008). The majority of previous studies focused on gene expression of *Pseudomonas* species used as a model to study plant–bacteria interaction in the rhizosphere. Barret et al. (2011) developed a list of 13 bacterial genes of *Pseudomonas spp*. induced in the rhizosphere in response to root exudates. These genes have functions in metabolism, motility, signal transduction, and unknown functions. Few studies have evaluated the gene expression in the rhizosphere soil. Some examples are: hydrogen cyanide (HCN) induces gene expression in the rhizosphere strawberry of a plant pathogen *Verticillium dahliae* (Decoste et al. 2010), gene expression of *Metarhizium anisopliae* that are induced by bean root exudates (Pava–Ripoll et al. 2011), expression of nifH genes by diazotrophic bacteria in the *Spartina alterniflora* rhizosphere (Brown et al. 2003), beta–propeller phytase gene expression in *Bacillus sp*. strain MQH15 in ryegrass rhizosphere (Jorquera et al. 2013), and quantification and characterization of genotypes of *Pseudomonas fluorescens* in rhizosphere soil based on 2,4–diacetylphloroglucinol (2,4–DAPG) genes (Mavrodi et al. 2007).

2.3.3 Microarray and transcriptome

The analysis of transcriptome profiles of rhizosphere strains by microarrays can give information on gene expression involved in the synthesis of several signals to control the bacterial activity in the rhizosphere (Wu et al. 2011). Tremblay and Déziel (2010) analyzed transcript profiles related to motility in *Pseudomonas aeruginosa*, a ubiquitous Gram–negative bacterium, by GeneChip® microarray. The results showed that swarming colonies displayed general down–regulation of genes associated with virulence and up–regulation of genes involved in energy metabolism. Van Puyvelde et al. (2011) studied the transcriptome of *Azospirillum brasilense* demonstrating that auxin indole–3–acetic acid is a signal molecule affecting its arsenal of transport proteins and cell surface proteins. However, microarray analysis relies on known genes from bacterial species; however, unknown genes are not detected. Therefore, this technique cannot provide information on the distribution and activities of unknown bacterial genes in the environment (van Elsas and Boersma, 2011). In this context, Shidore et al. (2012) analyzed the transcript profiles of *Azoarcus sp*. strain BH72, an endohpytic strain, exposed to root exudates released by *Oryza sativa*. The microarray analysis showed 2.4% and 2.0% of genes up–regulated and down–regulated, respectively; however, modulated gene expression included a few whose involvement in plant–microorganisms interaction had already been established, whereas a large fraction comprised of genes encoding proteins with putative or unknown functions.

2.3.4 Biosensors

Biosensors are defined as bacterial cells harboring a reporter gene, which is usually a fluorescence marker such as a green fluorescent protein (GFP) cassette expression (Sørensen et al. 2009). This system allows detection of activity and colonization of bacteria at the single cell level in rhizosphere microsites by epifluorescent and confocal

microscopy. Germaine et al. (2004) and Götz et al. (2006) successfully introduced GFP–tagged plasmids to localize and monitor the rhizosphere colonization of *Pseudomonas putida* strain PRD16, *Enterobacter cowanii* strain PRF116 and endophytic bacterial strains. Recently, Weyens et al. (2012) investigated the colonization and its capacity to promote plant growth by endophytic *P. putida* strain W619 with insertion of GFP–labelled, concluding that it did not promote growth. However, a limited number of reporter genes are available, high background fluorescence can limit detection, and the performance of biosensors can be variable depending on manipulation and sample preparation (Marschner et al. 2011).

2.3.5 Proteomics

Proteomics, defined as the entire protein complement expressed by a genome or by a cell, can be used for evaluating expression and localization of proteins, as well as for analysis of post–translational modifications (Wilkins et al. 1995). However, the extraction of intracellular proteins from soil is a methodological challenge, due to the stability of proteins (protected against proteolysis) and that they may be strongly adsorbed onto soil minerals or co–purify with humic acids or soil colloids that will interfere with analysis (Nannipieri, 2006, Arenella et al. 2014). According to Nannipieri (2006), intracellular protein–N that is the product of microbial activity accounts for an average of only 4% of the soil total N, whereas a value ranging from 30 to 50% is due to protein–N stabilized by surface–reactive particles. Therefore, the characterization of intracellular proteins should give insight into microbial functioning of the rhizosphere soil at the sampling time, whereas the characterization of the extracellular stabilized proteins should give indication on past microbial events. Keiblinger et al. (2012) compared four proteins extraction methods in two soils, the method with sodium dodecyl sulfate–phenol gave the highest yield. Recent studies have indicated that

proteins associated with metabolic functions such as carbohydrate catabolism and energy production, lipid and aminoacids biosynthesis, signal transduction, and membrane transport are the most abundant in rhizosphere and agricultural, potting, and forest soils (Damon et al. 2012; Wu et al. 2011; Lin et al. 2013). Usually, bacterial proteomics analyses are conducted by 2–dimensional polyacrylamide gel electrophoresis (2D–PAGE), 2D–difference gel electrophoresis (DIGE), and mass spectrometry (MS) for investigating expression gene detection involved between plant–pathogen, nitrogen–fixing bacteria in legumes and endosymbiotic interactions (Bestel–Corre et al. 2004; Cheng et al. 2010; VerBerkmoes et al. 2009). Another technique for the study of the ecology in rhizosphere bacteria is SIP (stable isotope probing)–protein technique with 13C or 15N that can be used for identifying bacterial populations involved in metabolic or biogeochemical processes in the bulk and rhizosphere soils (Bastida et al. 2009).

2.4. Post–genomic techniques in the study of bacteria in the rhizosphere

Currently, it is assumed that the development and improvement of techniques such as metagenomics, metaproteomics, and metatranscriptomics, will provide more accurate evaluation of the activities and compositions of microbial communities in rhizospheres than classical molecular techniques, generating new questions about the roles and functions of these microbial communities (Hirsch et al. 2010; Sørensen et al. 2009).

2.4.1 Metagenomics

In order to study and evaluate the richness, distribution and activity of microbial communities in bulk and rhizosphere soils, it is important to understand the ecological functions of each species. Modern molecular techniques have shown that bacterial diversity of bulk and rhizosphere soil is much greater than was predicted. Soil metagenome study of 16S rRNA gene and ITS1 region using next generation sequencing or second–generation sequencing technologies (Niedringhaus et al. 2011) have revealed that 1 g soil sample may contain 33,346 bacterial and archaeal OTUs (Mendes et al. 2011), 3,320 fungal OTUs (Schmidt et al. 2013), 145 to 200 of fungal OTUs (Xu et al. 2012), and 300 archaea OTUs (Pires et al. 2012). Both Roche 454 and Illumina platforms have been used to address the bulk and rhizosphere soils (Table 2.1). Roche 454 pyrosequencing platform produces long read length (<450 bp) and high consensus accuracy more than Illumina platform (Unno 2014). Uroz et al. (2010) by 454 pyrosequencing compared the bacterial diversity of oak rhizosphere and bulk soil, finding that Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes were the dominant taxa. Recently, Lagos et al. (2014) characterized the composition of bacterial communities of rhizosphere microsites (root tips and mature root zones) of Lolium perenne and found that Proteobacteria, Actinobacteria and Acidobacteria were the

dominant phyla. Similarly, Sun et al. (2014) characterized the diversity of bacterial communities of apple rhizosphere by Illumina sequencer and found that Proteobacteria, Acidobacteria, Bacteroidetes, Gematimonadates and Actinobacteria were the dominant groups.

Table 2.1 A summary of next generation sequencing platforms used in bacterial ecology studies

Company/ Platforms	Read lengh (bp)	Run time	Sequencing principle	Library amplification	Signal detection	System overview
Roche						
454 GS FLX Titanium XL+	~1000	23 h	Pyrosequencing by synthesis	Emulsion PCR on microbeads	Optical detection of light from pyrophosphate release on nucleotide incorporation	Single gene; genome and transcriptome sequencing
454 GS FLX Titanium	~600	10 h				
454 GS Junior	~400	10 h				
454 GS Junior+	~700	18 h				
Illumina						
Miseq	~300	~5–55 h	Reversible terminator sequencing by synthesis	Bridge PCR on flow cell surface	Optical detection of fluorescent emission from nucleotide incorporation	Single gene, genome sequencing
Hiseq 2500	~125–250	~7–60 h				Single gene; genome and transcriptome sequencing
Hiseq 3000	~150	1–3 days				
Hiseq 4000	~150	1–3 days				
Pacific Bioscience PacBio RS SMRT	20, 000	0.5–4 h	Single molecule real time sequencing by synthesis	Not required	Optical detection of fluorescent emission from labelled nucleotide incorporation	Single gene; genome and transcriptome sequencing

2.4.2 Metaproteomics

Metraproteomics provides a direct measure of proteins present in an environmental sample such as soil, offering information about the functional roles of soil microorganism, such as biogeochemical processes, degradation, or bioremediation processes (Bastida et al. 2012, Chourey et al. 2010). VerBerkmoes et al. (2009) proposed proteogenomics as an important tool for studying the physiology, ecology and evolution of microbial populations, communities and consortia in several environments as a means to connect a microbial species with its function. This approach is important since it combines metagenomics and proteomics and provides for the verification of metagenomics studies by protein data. However, it is necessary to considerer that the databases for soil protein identification are still incomplete. Nevertheless, numerous metaproteomics studies have revealed the diversity of proteins that are expressed by the interactions between plants and soil microbial communities. Recently, Lin et al. (2013) compared a metaproteomics profile of rhizosphere of ratoon sugarcane and plant sugarcane. The results revealed that ratoon sugarcane induced significant changes in the soil enzyme activities, the catabolic diversity of microbial community, and the expression level of soil proteins originated from the plants, microbes and fauna. This study also reported that 24.77% of soil proteins are derived from bacteria and most of the up–regulated expression of microbial proteins were involved to membrane transport and signal transduction. Similarly, Wu et al. (2011) realized a comparative metaproteomics analysis for determining the changes on soil protein abundance under consecutive monoculture of herb *Rehmannia glutinosa*. The results shown the identification proteins of plants, bacteria and fungi mainly involved in the carbohydrate and energy metabolism, aminoacids metabolism, stress/defense response. However, compared with Lin et al. (2013) study, lower percentages (11.65%) of proteins derived

from bacteria were found, mainly involved in signal transduction. Moreover, Moretti et al. (2012) demonstrated that the rhizosphere of lettuce (*Lactuca sativa*) produced higher amount of proteins related with virulence determinants, energy metabolism, stress/defense response in presence of pathogenic strain of *Fusarium oxysporum* which could be related with the interaction the microbial consortium associated to this fungus.

2.4.3 Metatranscriptomics

Metatranscriptomics involves the characterization of a set of messenger RNA (mRNA) (transcripts) produced in all cells, which would provide insight into the metabolic processes of a microbial community (Simon and Daniel, 2011; de Menezes et al. 2012). Consequently, metatranscriptomics analysis has the potential to discover novel genes and functions, allowing identification of active community members in both bulk and rhizosphere soils, and to correlate them with their metabolic activities (Kim et al. 2014). However, metatranscriptomic approaches have not been widely used in the rhizosphere, probably due to the instability of mRNAs and difficulties in their extraction from complex ecosystems. Among the more notable methodological challenges are their short half–lives, difficulties in the separation of mRNA from other RNA types (i.e. tRNA, rRNA miRNA) and interference from humic compounds that co–extract with nucleic acids from soil (Simon and Daniel, 2011). In addition, few studies have applied metagenomics, metaproteomics and metatranscriptomics due also to the difficulties and expense related to simultaneous extraction of nucleic acids and proteins from soil samples (Table 2.2). Moreover, the majority of studies in bulk and rhizosphere soils are mainly focused on bacteria, demonstrating the need for further studies to obtain further insights into the molecular ecology of other microorganisms present in rhizosphere microbiome, such as fungi, archaea, microalgae, protozoa, etc.

Table 2.2 Applications and limitations of post–genomic techniques currently used in soil Microbiology studies

Technique	Target	Application	Limitations	References
Metagenomics	DNA	Biodiversity and gene quantification	– DNA is extracted from all cells (active and non–actives) – Limited to the dominant members of the microbial community of soils	Bastida et al. 2009; Simon and Daniel, 2011; van Elsas and Boersma, 2011
Metatranscriptomics	RNA	Biodiversity and gene expression	– Liability of RNA – Interference by soil humic compounds	Simon and Daniel, 2011; Bastida et al. 2009
Metaproteomics	PROTEINS	Gene functions, activitiesand metabolic functions	– Intracellular proteins–N only 4% of total N – Prevalence of extracellular stabilized proteins–N not involved in the microbial activity at sampling but expression of past event – Strongly adsorbed onto soil minerals or humic colloids	Simon and Daniel, 2011; Bastida et al. 2009; Nannipieri, 2006

In order to better understand microbial genomes and characterize bacterial phylogeny, the Genomic Encyclopedia of Bacteria and Archaea Project (GEBA Project) was initiated in 2007. This project is lead by the US Department of Energy (USDOE) Joint Genome Institute, Institute of California Davis, USA, and German Collection of Microorganisms and Cell Cultures, Germany (DSMZ) (Wu et al. 2009). Currently, 200 bacterial genomes have been sequenced. The principal objectives of this project are to improve identification of proteins families, and to identify novel genes and undescribed organisms (http://goo.gl/FNpdwv revised in January, 2015). GEBA Project have other cooperative projects, such as GEBA–type strain, GEBA–RNB (Root Noduling Bacteria), and GEBA–MDM (Microbial Dark Matter). The main objective of GEBA–type strain project is to find novel functions of protein families, and thus, discovery of natural products. On the other hand, GEBA–RNB project is based in sequencing 100 symbiotic bacterial strains isolated from different soils around the world. This project can improve our understanding of the endosymbiotic relations between bacteria and roots, and nitrogen fixation. The GEBA–MDM project objective is to use single–cell genomics to explore uncultured bacteria and archaea that GEBA project does not include. Hence, the discovery of novel genomes by this project will improve phylogeny and the evolution of bacterial and archaeal domains (Rinke et al. 2013).

Finally, the Pacific Biosciences single–molecule real–time (PacBio RS SMRT) sequencer is a third–generation sequencing technology (Niedringhaus et al. 2011). It is noteworthy that the PacBio RS SMRT has been applied to genome sequencing of *Streptomyces sp.* strain Mg1, a soil bacterium can cause lysis and degradation of *Bacillus subtilis* (Hoefler et al. 2013). Moreover, it may be a suitable platform for longer sequencing reads of 16S rRNA genes from environmental samples; according to

Mosher et al. (2013), this approach could provide more information about microbial taxonomy and the phylogeny of microbe inhabitants of the rhizosphere.

2.5. Conclusions and perspectives

Rhizospheres are complex and dynamic habitats characterized by high microbial activities. The diversities in different zones in the rhizosphere can be influenced by plant–bacteria and bacteria–bacteria interactions regulated by communication, competition along root zones, plant growth stage, and plant species. The advances in soil molecular and post–genomic techniques will continue to improve our understanding of the compositions and activities of soil microbial communities, to target the rare or low abundance bacterial populations in the rhizosphere, and to predict in situ responses, activities, and growth of bacterial communities. Until very recently, few studies of soil and rhizosphere soils have been performed using metagenomics, metaproteomics and metatranscriptomics; these approaches may be limited by difficulties of extractions and purification of nucleic acids and proteins from complex environmental samples. Advances in nucleotide and protein sequencing techniques might allow lower costs, making them more accessible, expanding libraries DNA or database to continue uncovering more genomes and functions of microbiota of bulk and rhizosphere soils. Being an interesting approach for improving strategies to select indigenous potentially beneficial bacterial strains that may function as biofertilizers or bioprotectors, to understand the importance of the rhizosphere microbiome in promoting plant health and, to study the mechanisms of plant impacts (exudates) on the rhizosphere soil microbiome (Bakker at al. 2013). Therefore, collaborative efforts are needed for future biotechnological developments allowing the establishment of adequate and efficient

strategies of rhizosphere management, benefitting the yield and sustainability of agricultural systems.

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CHAPTER 3

Bacterial community structure in rhizosphere microsites of ryegrass (*Lolium perenne* var. Nui) as revealed by pyrosequencing

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Bacterial community structure in rhizosphere microsites of ryegrass (*Lolium perenne* var. Nui) as revealed by pyrosequencing

Abstract

Management of soils to facilitate plant beneficial microbial interactions requires basic knowledge of the species composition and microbial community structures in the plant rhizosphere. Here, we examined bacterial communities associated with in rhizosphere microsites located at the root tips and mature root zones of *Lolium perenne* when grown in Chilean ash-derived volcanic soils (Andisols: Freire and Piedras Negras soil series). Community structures were analyzed by pyrosequencing of 16S rRNA genes followed by in silico analysis for phylogenetic assignments (MOTHUR and VITCOMIC). Analysis of the community structure revealed significant differences in community structures in relation to the soil series, which differed particularly in the relative abundance of Cyanobacteria and Firmicutes. However, no significant differences were observed with respect to root microsite location in the same Andisol series. Predominant taxa included members of the Proteobacteria, Actinobacteria and Acidobacteria. Analysis by VITCOMIC showed that dominant bacterial groups comprised only 5 to 10% of the total bacterial community and the remaining majority of bacteria low abundant taxa (Fusobacteria, Thermotogae, Lentisphaerae, Tenericutes, Deferribacteres Spirochaetes, Planctomycetes, Thermotogae, Deinococcus-Thermus), most of which have not been previously reported or associated with the plant rhizosphere according to GenBank database. The results indicate that most of bacteria in Chilean Andisols have not been described to the rhizosphere plants and their functional traits are still largely unknown

3.1. Introduction

The plant rhizosphere is a complex environment where diverse microorganisms contribute to plant nutrition, hormonal control of plant root growth, and disease suppression (Bastida et al. 2009). Given these important functions, there is considerable interest in understanding the linkage between microbial community structures, soil management practices, and plant growth in different types of soils. Early studies on the rhizosphere mainly relied on culture based techniques and low resolution molecular methods such as PCR-DGGE or PLFA profiles of microbial community structure, which revealed that rhizosphere communities vary in relation to plant species and soil type. Rhizosphere communities also are highly dynamic spatially and temporally, reflecting changes in the abundance and chemical composition of root exudates that serve as carbon and energy sources for microorganisms (Bertin et al. 2003), as well as changes in abiotic factors (pH, organic matter, soil type, etc.) that affect the selection of different microbial species (Chaparro et al. 2012; Hannula et al. 2014; Marschner et al. 2011; Nannipieri et al. 2008a, 2008b). Rhizosphere communities have been shown to vary along the root axes in relation to the concentrations and types of labile carbon that are deposited into the rhizosphere. According to current models, labile carbon is greatest in the zone of elongation. Changes in labile carbon along the root axes are postulated to drive a successional process in which the distal zone selects for growth of opportunistic copiotrophic bacteria, which are then followed by a succession of other microbial species that are adapted to an increasingly crowded and carbon limited environment in the older root zones (Marschner *et al*. 2011; Yang and Crowley 2000).

With the advent of pyrosequencing methods, it has now become possible to re-examine the structure, diversity and activity of microorganisms at much higher

resolution and in more detail and for different types of plants in different soils. These high resolution analyses of the taxonomic composition of the rhizosphere provide baseline information on the specific microflora that occur in the rhizosphere, from which we can thereafter deduce the communities structures at specific root zones, and their functional relationships with plant growth, nutrient uptake, and root health. One of the long term goals of rhizosphere biology research is to understand how specific soil factors and management practices affect plant productivity and disease. Much attention has also been paid to plant growth-promoting bacteria (PGPR), many of which are Alpha and Gammaproteobacteria that are favored by high pH. However, the acquisition of high resolution data on the taxonomic compositions of soil microbial communities and plant-microbial associations has just begun and will require extensive research to obtain fundamental information on community structures and their functional properties.

Here, we report the first description of the bacterial composition of the rhizosphere for perennial ryegrass (*Lolium perenne* L.) in Chilean volcanic soils (Andisols). These soils comprise 43% of the total agricultural land in southern Chile, and are widely used for pasture for beef cattle and dairy milk production in Southern Chile (Mora et al. 1999). Previously, our group has studied the microbiology of these soils using both culture-dependent and culture-independent methods (Jorquera et al. 2008, 2010 and 2011; Martínez et al. 2011), with the aim of identifying and isolating plant growth promoting rhizobacteria (PGPR) that may be used as biofertilizers. However, our understanding of the ecology of these bacteria, and their interactions with indigenous microbial communities will ultimately proceed only through comprehensive understanding of bacteria-bacteria interactions (colonization, competition, communication and others) at specific root microsites for plants grown under defined

environmental conditions. In this study, we examined the structure of bacterial communities by pyrosequencing of 16S rRNA genes from DNA extracts of soil associated with selected microsites of the rhizosphere of ryegrass plants (*Lolium perenne* var. Nui) grown in two Chilean Andisols. Andisols were chosen based on agronomic managements, naturalized pasture without any fertilizer application for >5 years (Andisol Freire series) and pasture under periodical fertilization (Piedras Negras series).

3.2. Material and methods

3.2.1 Soils

Two Chilean Andisols under long term pasture were chosen for culture of ryegrass plants in root boxes used for collection of roots and rhizosphere samples under defined conditions. The two soils represent two of the major series in this region and included a soil from the Freire series (38°50′S 72°41′W) and another from the Piedras Negras series (40°20'S and 72°35'W). The soils were collected from the surface a horizon at 0-20 cm depth, after which they were transported to the laboratory, passed through a 2 mm sieve and stored in a refrigerator until their use for plant culture. Soil chemical were analyzed. Briefly, pH was measured in 1:2.5 suspension of soil in deionized water. Available P (POlsen) was extracted using 0.5 M bicarbonate (pH 8.5) and analyzed by the molybdate−blue method (Murphy and Riley 1962). Organic matter was estimated by wet digestion (Walkley−Black procedure). Exchangeable cations (K+, Ca2+, Mg2+, and Na+) were extracted with 1 M ammonium acetate at pH 7.0 and analyzed by flame atomic adsorption spectrophotometry (FAAS) (Warncke and Brown 1998). Exchangeable Al was extracted with 1 M KCl and analyzed by FAAS (Bertsch and Bloom 1996). The chemical properties of the two Andisols are shown in Table 3.1

Table 3.1. Some chemical characteristics of two Andisols used in this study

	Andisol		
	Freire series	Piedras series	Negras
pH $_{H2O}$	5.68	5.43	
P_{Olsen} (mg kg^{-1})	19	2	
Organic matter (%)	12	15	
K [$cmol_{(+)}$ kg^{-1}]	0.39	0.13	
Na [$cmol_{(+)}$ kg^{-1}]	0.14	0.12	
Ca [$cmol_{(+)}$ kg^{-1}]	6.63	2.2	
Mg [$cmol_{(+)}kg^{-1}$]	0.92	0.59	
Al [$cmol_{(+)}kg^{-1}$]	0.12	0.3	
CEC [$cmol_{(+)}$ kg-^{1}]	8.2	3.05	
Σ Bases [$cmol_{(+)}kg^{-1}$]	8.08	3.05	
Al saturation (%)*	1.46	8.84	

3.2.2 Rhizotrons

Root boxes were built according to the design described by Wang *et al*. (2004) (Figure 3.1A). The rhizotrons were 25 cm long, 12 cm wide and 2.5 cm deep, and were constructed of black plastic with a removable glass plate on one side that was covered with a dark plate to exclude light during plant growth. Triplicate rhizotrons were prepared for each Andisol. After packing with soil, the rhizotrons were placed in a support frame to maintain each root box cassette at a 30° angle to direct root growth toward the glass sides of the boxes. The ryegrass (*Lolium perenne* var. Nui) plants were introduced by planting presterilized (treatment for 3 min in a solution of 5% v/v sodium hypochlorite and rinsed with six changes of sterile demineralized water for 30 min) seeds. Ten seeds per rhizotron were planted at 2.0 cm depth and rhizotrons were placed in a growth chamber at 20ºC for 30 days with a photoperiod 16:8 h light: dark, PAR (photosynthetically active radiation) of 300 μmol photons m-2 s-1 and relative humidity of ~60%.

3.2.3 Rhizosphere microsite sampling

After 30 d, two rhizosphere microsites for the analysis were selected: root tips and mature zone (Figure 3.1A). Samples (0.5-1 cm of root) of both microsites were separately obtained from 2-3 plants for each rhizotron (in triplicate) (Figure 3.1B) and soil aggregates were detached from roots by vigorous vortexing and collected in sterile polypropylene microtubes. Posteriorly, root pieces were discarded and soil rhizosphere samples were gently mixed until reach 1-2 g of rhizosphere soil samples and subjected to DNA extraction.

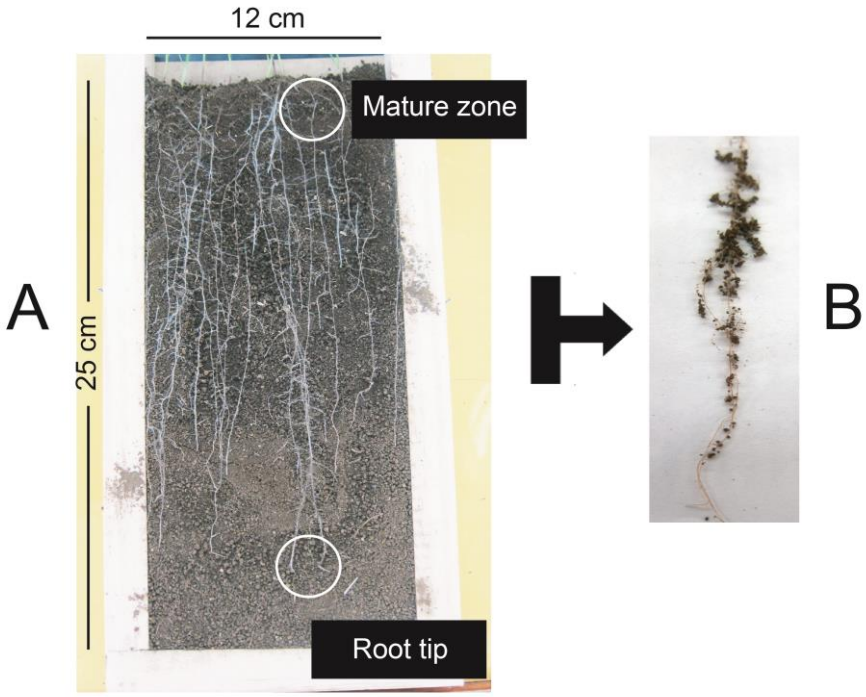

Figure 3.1 (a) Rhizotron system used in this study and showing the rhizosphere microsites sampled (root tip and mature zone). (b) Root-attached soil aggregates used for DNA extraction

3.2.4 Total DNA extraction and Pyrosequencing

Total DNA was extracted from rhizosphere soil samples with a Power Soil® DNA Isolation Kit (MoBio Inc., USA) and Powelyzer® homogenizer (MoBio Inc., USA)

according to the manufacturer's instructions. DNA quality and quantity were measured using spectrophotometer (Thermo Scientific Multiskan GO UV/Vis model). DNA purity was assessed by determination of an A280/A260 absorbance ratio > 2.0. Purified DNA extracts were precipitated with 2.5 vol of ethanol (100%), and then resuspended to a concentration of 15 ng μL-1 in 20 μL of TE buffer. The purified samples were then submitted to Macrogen, Inc. (Seoul, Korea) for pyrosequencing analysis. The genomic DNA was amplified separately by PCR using the bacterial primer pair UNI_AMP-27F (5'-AxxxG AGT TTG ATC MTG GCT CAG-3') and UNI_AMP-518R (5'-BWT TAC CGC GGC TGC TGG-3') to genera 16S rRNA fragments (bp), where A and B represent two pyrosequencing primers (CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG and CCT ATC CCC TGTG TGC CTT GGC AGT CTC AG) and xxx was designed for the sample identification barcoding key. The libraries were sequenced by using Roche 454 GS-FLX System using Titanium Chemistry (454 Life Sciences).

3.2.5 Pyrosequencing data

The sequence data were processed and analyzed by program, MOTHUR version 1.27 (http://www.mothur.org/) (Schloss et al. 2009). The reduction of sequences de-noising, filtering of low-quality reads, trimming of the barcode sequences, align and detection of chimeras with UCHIME (Edgar et al. 2011) were carried out according to standard operational procedures (http://www.mothur.org/wiki/Schloss_SOP; accessed February, 2014). After filtering, sequences were clustered into species-level operational taxonomic units (OTUs) at the 3% dissimilarity cut-off and the amount of sequences by each group was rarified according to the group with lowest quantity of them. Each OTU was assigned to the species level, using a naive Bayesian classifier provided by the SILVA 16S rRNA database at a confidence threshold of 0.8. The sequences were aligned against those sequences deposited in the SILVA database using the kmer search

tool to find template sequences and Needleman-Wunsch algorithm to align them. Sequences exhibiting at least 96.95% sequence similarity over at least 286 pb were assigned to each species. Good's coverage (Good, 1953) was used to evaluate the sequence coverage.

The sequences obtained in this study are available at the NCBI Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under accession number SRX339430 and compared with those present in SILVA databases by using SINA aligner (http://www.arb-silva.de/aligner/) (Pruesse *et al*. 2012).

3.2.6 Alpha- and beta-diversity analysis

Alpha-diversity indexes were estimated based on the observed number of OTUs. For each rhizosphere microsite sample, relative abundances were determined by dividing the number of reads for any given OTU for the total number of reads obtained. Chao1 indexes were calculated and rarefaction curves were also depicted to assess the species richness, by plotting the estimated number of OTUs as a function of the number of sampled individuals.

In relation to Beta-diversity indexes, Jaccard similarity (coefficient based on the observed richness in the community membership, presence/absence), and Yue & Clayton theta similarity (coefficient based on the observed abundance in the community structure) were used and the diversity estimators were visualized using a heat map. Community distances between each sample were calculated by unweighted UniFrac. Analysis of molecular variance (AMOVA) was used to test differences in the bacterial species diversity in relation to microsite location and soil series.

3.2.7 VITCOMIC analysis

Structures of the bacterial communities for the two rhizosphere microsites were visualized and compared using VITCOMIC software (VIsualization tool for Taxonomic COmpositions of MIcrobial Community), which can analyze millions of bacterial 16S rRNA sequences. Moreover, VITCOMIC calculates the overall taxonomic composition for a microbial community, relative abundance and diversity by Jaccard, Lennon, Yue and Clayton theta indices (Mori et al. 2010).

3.3. Results

3.3.1 Soils

Soil chemical analyses showed that the Freire and Piedras Negras soils that were used for the analysis of rhizosphere community structures mainly differed in available P (19 and 2 mg kg-1 of P, respectively) and percentage of Al saturation (1.46 and 8.84%, respectively) (Table 3.1).

3.3.2 Pyrosequencing data

The 16S rRNA gene data yielded a total 201,645 reads ranging from 35,000 to 60,000 for each sample, with an average length of 286 bp and an average value of Good's coverage of 90% calculated by MOTHUR, which is considered as sufficient for the analysis of bacterial composition (Table 3.2). The number of high-quality sequences was rarified to 21,157 sequences and used for further analysis.

Table 3.2. Total reads and number of sequences identified in this study at different operational taxonomic unit (OTU) levels.

	Freire serie		Piedras Negras serie	
	Root tips	Mature zone	Root tips	Mature zone
Total n° reads	61,975	77,359	51,607	87,412
Total n° no hit reads	2,389	1,411	693	2,028
OTU				
Phylum	61,129	76,125	51,027	86,154
Order	36,531	53,040	39,506	66,072
Genus	20,000	32,895	24,988	42,607

3.3.3 Alpha- and beta-diversity analysis

3.3.3.1 Alpha diversity analysis

At the phylum level species belonging to the Proteobacteria, Actinobacteria and Acidobacteria were the dominant taxa in both Andisols (Figure 3.2). Within the Proteobacteria (30-32% of relative abundance), members of the class Alphaproteobacteria were the most abundant (10.6-16.2×103 reads) followed by Betaproteobacteria (3.6-11.6×103 reads), Gammaproteobacteria (1.1-4.6×103 reads) and Deltaproteobacteria (1.2-2.4×103 reads). Within the Actinobacteria (19-27% relative abundance), members of the order Actinomycetales were the most abundant (6.6-11×103 reads) followed by Solirubrovacterales (1.6-2.2×103 reads), and Acidimicrobiales (1.1-1.7×103 reads). Within Acidobacteria (8-14% of relative abundance), members of order Acidobacteriales were the most abundant (1.7-3.4×103 reads). Comparison of the phylum-based community structures suggested differences in the relative abundance of the major phyla in the two Andisol series (Figure 3.2). Independent of root microsites, there was a higher relative abundance (17-23%) of Cyanobacteria in the bacterial community associated with ryegrass grown in the Freire

series compared to Piedras Negra series (3-4%). In contrast, a higher relative abundance (8-10%) of Firmicutes was observed in the rhizosphere samples in the Piedras Negras series compared with Freire series (1%). Similarly, Chloroflexi and Bacteroidetes represented a higher relative abundance (4-5%) in the Piedras Negras series as compared to the Freire series (1-2%). At the genus level, the pyrosequencing data showed that members of the taxa, Bradyrhizobium (Alphaproteobacteria), Pseudomonas (Gammaproteobacteria) and Bacillus (Firmicutes) were the most abundant with relative abundances >5% in all microsites. However, the majority of the identified bacterial genera were of low relative abundance, comprising < 5% of the total reads.

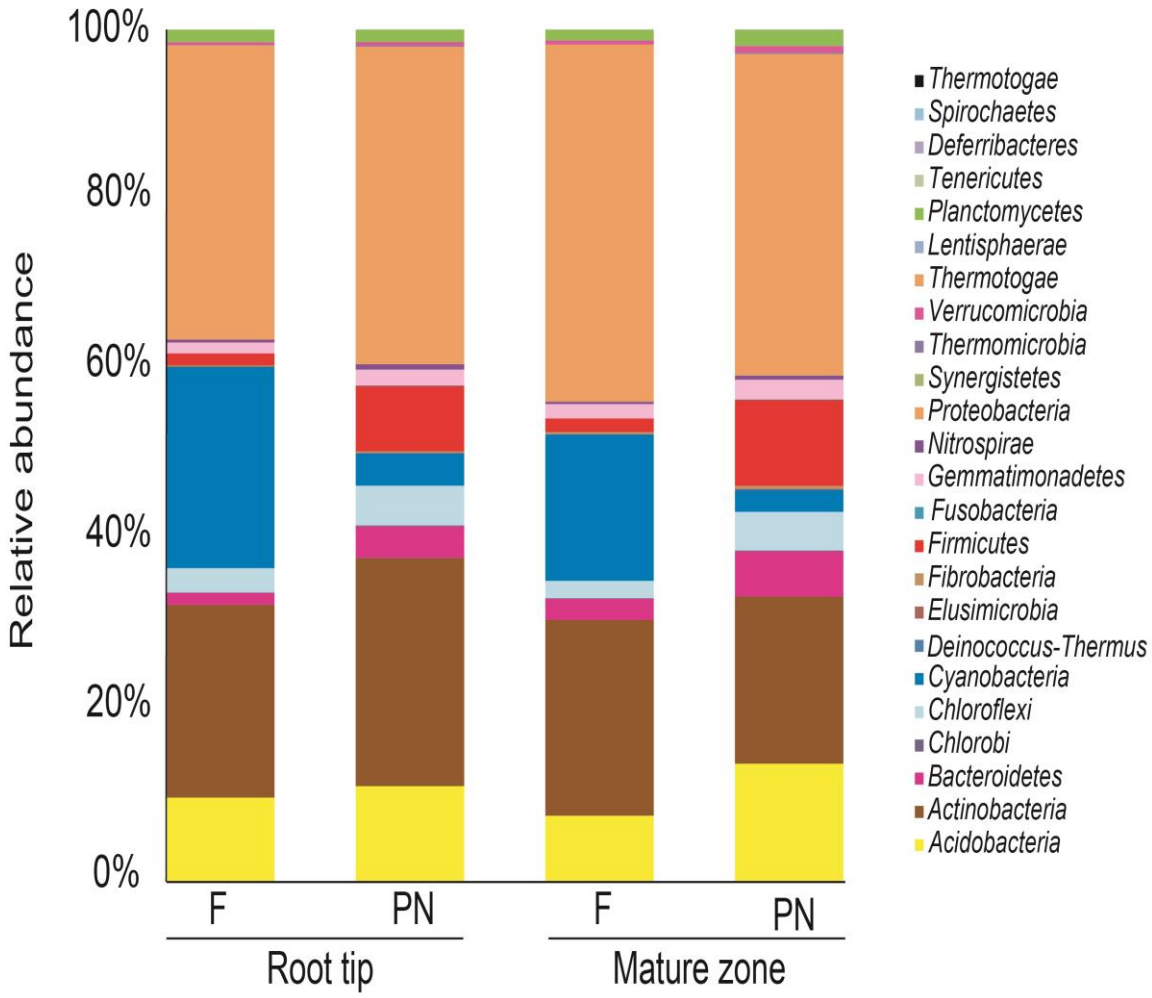

Figure 3.2. Relative abundance of phyla in different rhizosphere microsites (root tip and mature zone) of *Lolium perenne* var. NUI grown in Freire (F) and Piedras Negras (PN) series.

In relation to bacterial community structure, the estimations of species richness (number of species present) were similar for all samples, the observed number of OTUs showed some small variation among samples (Table 3.2). The highest total richness estimate using Chao1, was obtained for the mature root zones and root tips for plants grown in Freire soil series, which had 11,005 and 9,964 OTUs respectively, followed by the mature zones and root tips of plants grown in the Piedras Negras series, which had 9,707 and 9,629 OTUs, respectively (Figure 3.3A). For all of the samples, independent from the niche, non-parametric estimators of diversity indicated that total OTU richness was much higher than the currently observed in our study.

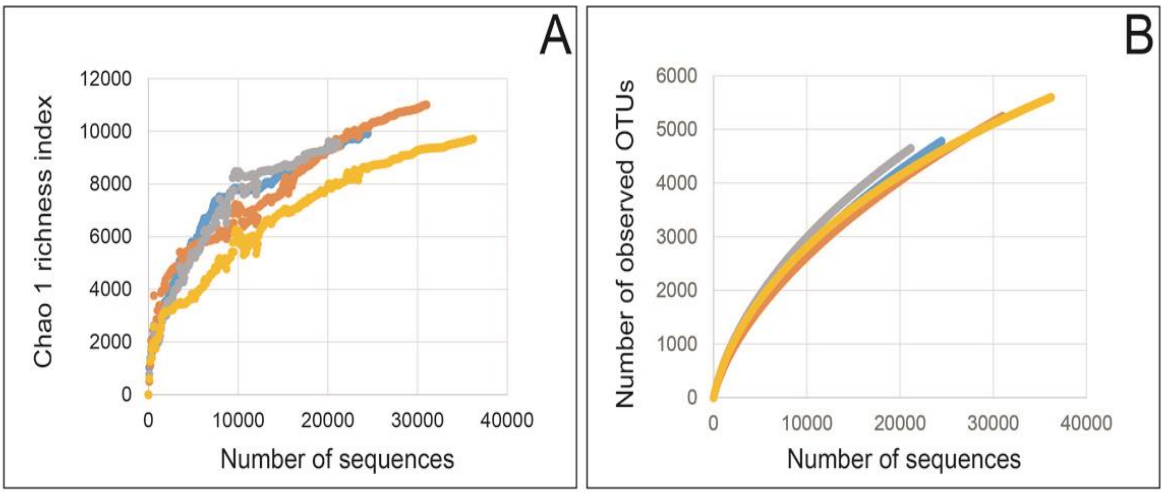

Figure 3.3 (a) Chao1 index showing the richness of rhizosphere microsites of *Lolium perenne* grown in Freire and Piedras Negras series. (b) Rarefaction curves showing the dependence of discovering novel OTUs as a function of number of sequences for OTUs defined at a 0.03 distance cut-off. Lines color corresponds to rhizosphere microsites as follow: orange: Freire series-root tip; blue: Freire series-mature zone; yellow: Piedras Negras series-root tip; gray: Piedras Negras series-mature zone.

Rarefaction curves were generated for the four samples with a 0.03 distance cut-off (Figure 3.3B). Rarefaction curves provide a means for comparing the diversity observed in the different samples. Moreover, these curves describe the dependence of discovering novel OTUs as a function number of sequences. Rarefaction curves obtained with 21,000-36,200 sequences suggested that with increased number of sequences, the samples likely would reach the plateau phase. The curves for root tip and mature zone in Freire series ascend to 4,779 and 5,242 OTUs when 24,460 and 30,995 sequences were analyzed, respectively. The curves for root tip and mature zone in Piedras Negras series ascend to 4,647 and 5,596 OTUs when 21,158 and 36,200 sequences were analyzed. These results showed no differences between the rhizosphere samples. Consequently, there are no differences between the values for richness of OTUs obtained for the considered environments.

3.3.3.2 Beta –diversity analysis

Similarities in community membership (presence/absence) were compared using the traditional Jaccard similarity coefficient based on observed richness. The visualization by heat map showed the similarity values, whereas closer to number one communities are more similar (Figure 3.4A). Some of the dissimilarity could be explained by the higher relative abundance of Cyanobacteria in communities associated with ryegrass grown in the Freire series as compared to plants grown in Piedras Negras series. In contrast, a higher relative abundance of Firmicutes communities was associated with ryegrass grown in the Piedras Negras series compared with Freire series. To compare similarities in community structure based on relative abundance, we used the Yue Clayton & Theta similarity coefficients. These data are presented as a heat map which shows the similarity values, in which communities having values closer to number one, communities are more similar (Figure 3.4B). Distance measure between bacterial

communities using phylogenetic information by unweighted Unifrac showed 2% dissimilitude among communities (Figure 3.4C). Accordingly, the community structures are similar among root tip and mature zone (AMOVA p=0,331).

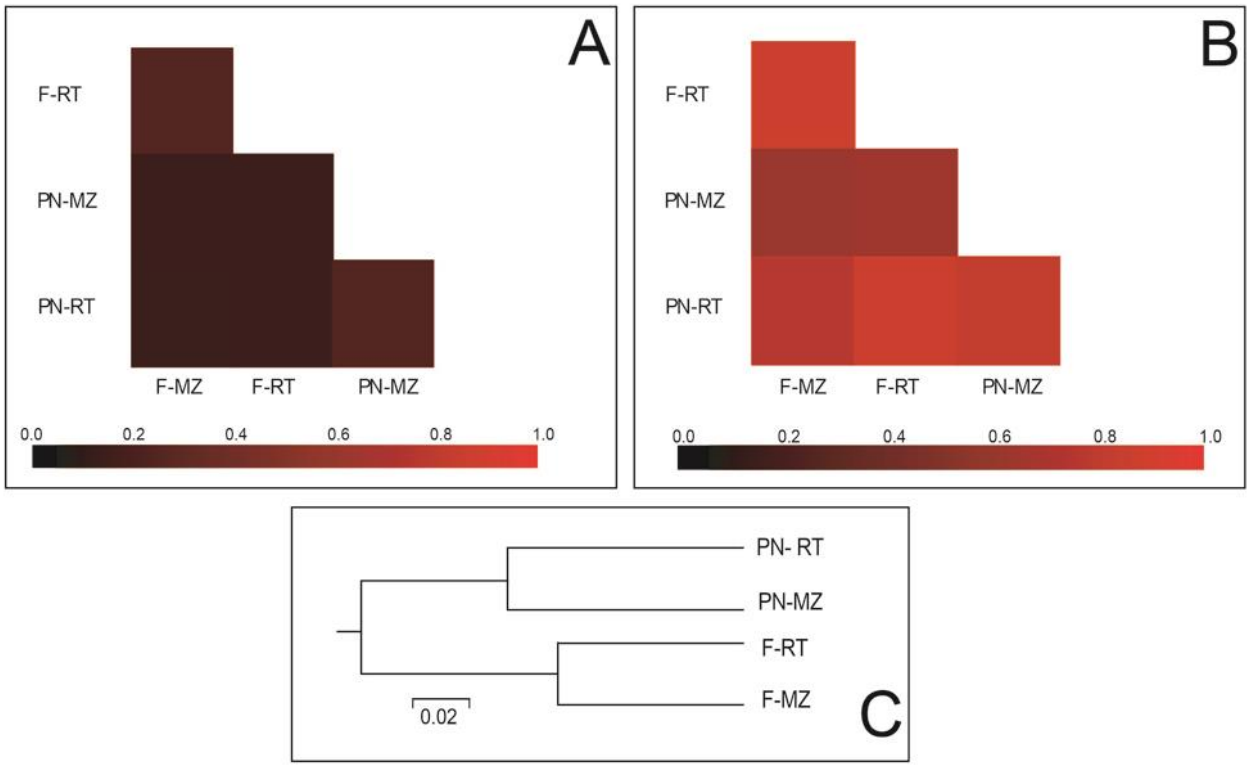

Figure 3.4. Visualization of Jaccard (a) and Yue Clayton & Theta (b) similarity coefficients by heat-map. (c) Phylogenetic distance between bacterial communities revealed by unweighted UNIFRAC. F-RT: Freire series-root tip; F-MZ: Freire series-mature zone; PN-RT: Piedras Negras series-root tip; PN- MZ: Piedras Negras series-mature zone.

3.3.4 VITCOMIC analysis

High resolution analysis of the OTU sequence data for the bacterial community structures at the species level was carried using VITCOMIC software based on 601 reference 16S rRNA gene sequences. This in silico analysis confirmed that the members of phyla, Proteobacteria and Cyanobacteria had the highest relative abundance in all of the communities that were analyzed. The VITCOMIC mapping clearly showed relative abundance of bacterial species in each rhizosphere microsite sampled but with low abundance for each one. Thus, around of 50% of genera (among 430-554 identified genera) in the rhizosphere communities had a relative abundance <1%, and 99% of the genera had a relative abundances <5%. Proteobacteria and Firmicutes showed the highest richness in all of the rhizosphere communities. Comparisons of the bacterial community structures across rhizosphere microsites (root tip and mature root zone) for each Andisol (Figure 3.5 and 3.6) indicated no significant difference in community structures based on indexes of Jaccard (0.59 and 0.65), Lennon (0.75 and 0.85), and Yue and Clayton theta (0.88 and 0.85) for the Freire and Piedras Negras series, respectively (Figure 3.7). These data visualized by the VITCOMIC software as gray dots represent common taxa occurring at both microsites analyzed.

It is noteworthy that the analysis with the SINA aligner revealed the occurrence of 79 genera that have not been previously reported for soil or rhizosphere environments according to the descriptions of these genera in the GenBank database (Table 3.3). However, the comparison with reference sequences in SILVA database showed low percentage similarities (2-32%). These genera included bacteria described as biopolymer-degraders, sulfate-reducers, alkane-degraders, oil-degraders, etc.), which were isolated from diverse habitats, including sediments and waters from sea and lakes,

the gastrointestinal tract and gut of humans and animals, industrial sludges, sedimentary rocks, cave, glacial, and other environments.

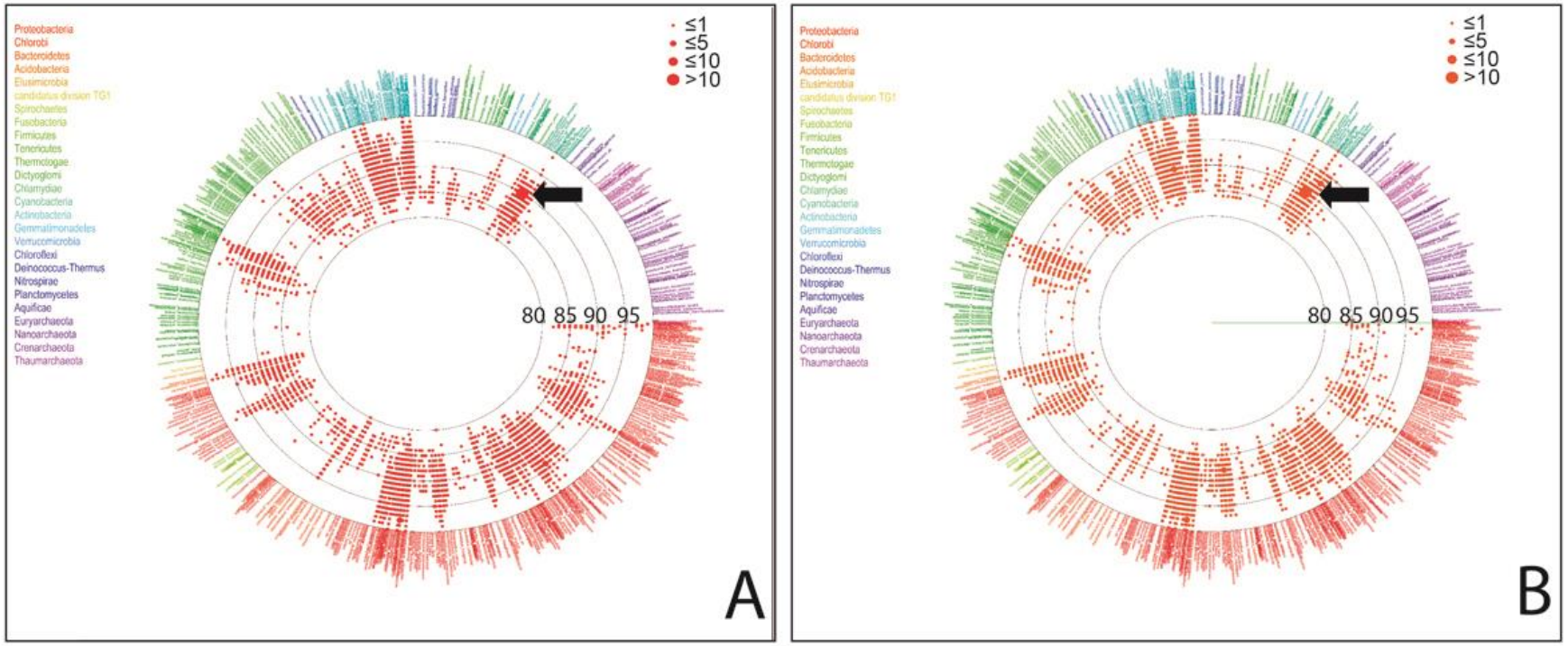

Figure 3.5 Mapping results by using VITCOMIC software for relative abundance of bacterial species (based on 16S rRNA gene) in root tip (a) and mature zone (b) from *Lolium perenne* rhizosphere grown in Freire serie Andisol. The relative abundance is represented as a circle plots and circle size indicates relative abundance of sequences in the bacterial community (from the smallest dot ≤ 1%, ≤5, ≤10 and > 10%). Black arrows denote bacterial groups with highest relative abundance (≤10%) within community. Each species name is placed outside the most lateral circle and the font color for each species name corresponds to its phylum name: red shades: *Proteobacteria*, *Chlorobi*; orange shades: *Bacteroidetes*,*Acidobacteria*, *Elusimicrobia*, candidates division TG; green shades: *Spirochaetes*, *Fusobacteria*, *Firmicutes*, *Tenericutes*, *Thermotogae*, *Dictyoglomi*, cyan shades: *Cyanobacteria*, *Actinobacteria*, *Gemmatimonadetes*, blue shades: *Verrumicrobia*, *Chloroflexi*, *Deinococcus- Thermus*, violet shades: *Nitrospirae, Planctomycetes, Aquificae*. Large circles indicate boundaries of blast average similarities (80, 85, 90, 95 and 100% similarity of the VITCOMIC database sequence).

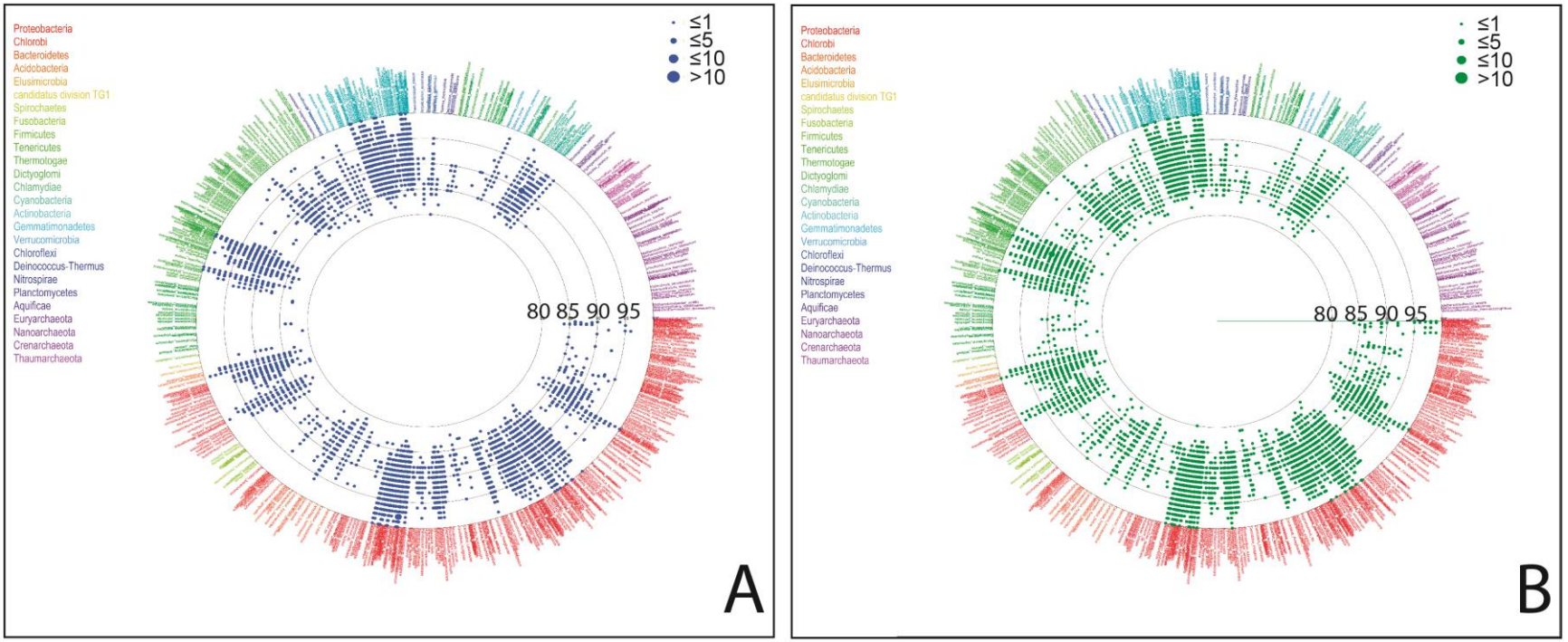

Figure 3.6 Mapping results by using VITCOMIC software for relative abundance of bacterial species (based on 16S rRNA gene) in root tip (a) and mature zone (b) from *Lolium perenne* rhizosphere grown in Piedras Negras serie The relative abundance is represented as a circle plots and circle size indicates relative abundance of sequences in the bacterial community (from the smallest dot ≤ 1%, ≤5, ≤10 and > 10%). Each species name is placed outside the most lateral circle and the font color for each species name corresponds to its phylum name: red shades: *Proteobacteria, Chlorobi*; orange shades: *Bacteroidetes*,*Acidobacteria*, *Elusimicrobia*, candidates division TG; green shades: *Spirochaetes*, *Fusobacteria*, *Firmicutes*, *Tenericutes*, *Thermotogae*, *Dictyoglomi*, cyan shades: *Cyanobacteria*, *Actinobacteria*, *Gemmatimonadetes*, blue shades: *Verrumicrobia*, *Chloroflexi*, *Deinococcus- Thermus*, violet shades: *Nitrospirae*, *Planctomycetes*, *Aquificae*. Large circles indicate boundaries of blast average similarities (80, 85, 90, 95 and 100% similarity of the VITCOMIC database sequence).

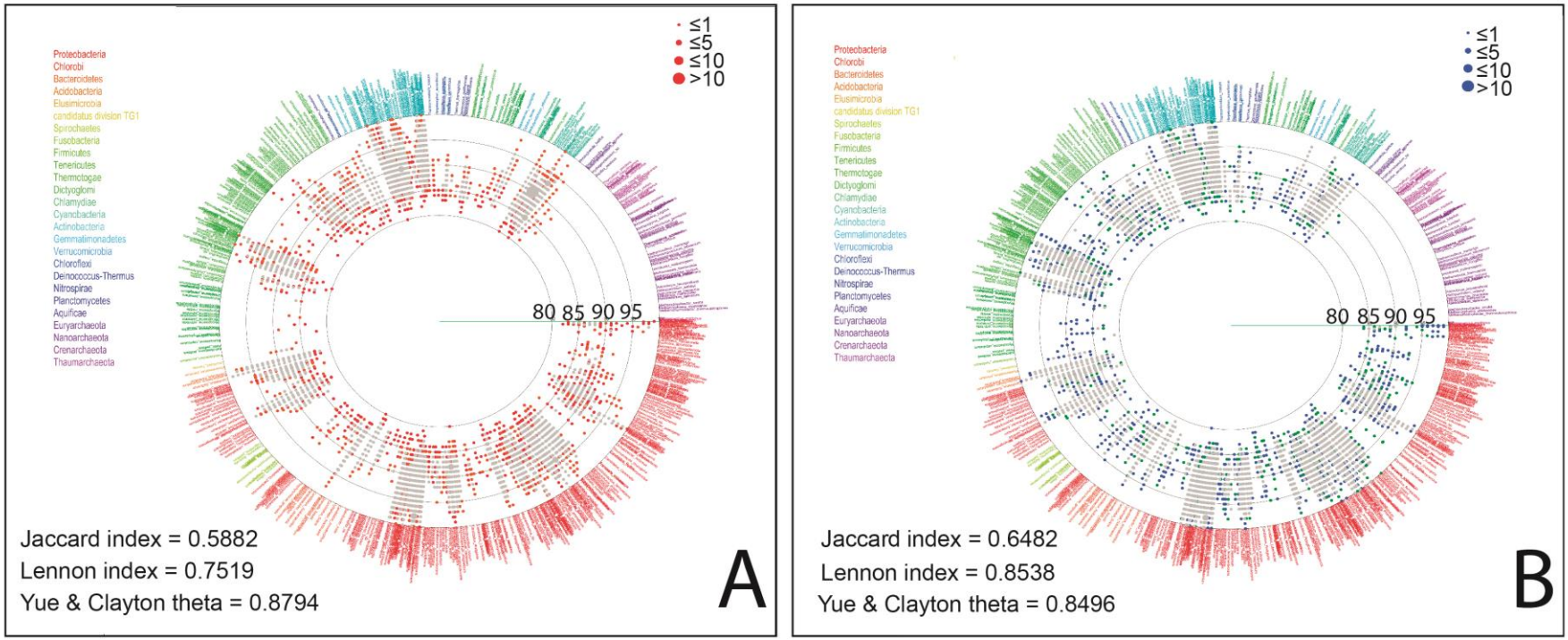

Figure 3.7 Comparison of 'root tip versus mature zone' by using VITCOMIC software of bacterial communities (based on 16S rRNA gene) in *Lolium perenne* rhizosphere grown in Freire serie (a) and Piedras Negras serie (b) Andisols. The relative abundance is represented as a circle plots and circle size indicates relative abundance of sequences in the bacterial community (from the smallest dot ≤ 1%, ≤5, ≤10 and > 10%). Gray dots indicate common taxa between analyzed communities. Each species name is placed outside the most lateral circle and the font color for each species name corresponds to its phylum name: red shades: *Proteobacteria, Chlorobi*; orange shades: *Bacteroidetes*,*Acidobacteria, Elusimicrobia,* candidates division TG; green shades: *Spirochaetes, Fusobacteria, Firmicutes, Tenericutes, Thermotogae, Dictyoglomi*, cyan shades: *Cyanobacteria, Actinobacteria, Gemmatimonadetes*, blue shades: *Verrumicrobia, Chloroflexi, Deinococcus- Thermus*, violet shades: *Nitrospirae, Planctomycetes, Aquificae*. Large circles indicate boundaries of blast average similarities (80, 85, 90, 95 and 100% similarity of the VITCOMIC databa sequence

Table 3.3 Phylogenetic assignment of bacterial genus found in this study, which have not been previously described or associated with the rhizosphere.

Taxonomic group[a]	**Genus of closest relatives or cloned sequences (accession no.)**[b]	**Similarity (%)**[b]	**Source isolation (activity)**
Synergistetes; Synergistia; Dethiosulfovibrio	*Dethiosulfovibrio peptidovorans* (U52817)	2.5	Thiosulfate-reducing bacterium, offshore oil wells
Deinococcus-Thermus; Deinococci	*uncultured bacterium* KD3-62 (GU454916)	30.9	Waste activated sludge
Bacteroidetes; Flavobacteria; Vitellibacter	*Vitellibacter* NCAAH 30A6 (GQ221072)	26.3	Water and sediment from a tropical brackish water
Bacteroidetes; Flavobacteria; Capnocytophaga	*Capnocytophaga cynodegmi* (EU122391)	33	Human intestine
Bacteroidetes; Flavobacteria; Cloacibacterium	*Cloacibacterium, uncultured bacterium* (GQ111742)	3.2	Human skin
Proteobacteria;Gammaproteobacteria; Haliea	uncultured bacterium (FJ715969)	25.1	Intestinal tract (*Lumbricus rubellus*)
Proteobacteria;Gammaproteobacteria; Methylosoma	uncultured bacterium (GQ500833)	28.4	Cave sediment
Proteobacteria;Gammaproteobacteria; Pseudospirillum	uncultured bacterium (GQ263860)	26.6	Waste
Proteobacteria;Gammaproteobacteria; Rugamonas	*Rugamonas rubra* (HM038005)	2.2	n.i
Proteobacteria;Gammaproteobacteria; Thioalkalispira	uncultured bacterium (FJ717190)	23.7	Marine sediment
Proteobacteria;Gammaproteobacteria; Thiorhodococcus	*Thiorhodococcus minor* (FN293057)	23,2	n.i
Proteobacteria;Gammaproteobacteria; Actinobacillus	uncultured bacterium (GQ116320)	3	Human skin
Proteobacteria;Gammaproteobacteria; Crenothrix	uncultured *Methylobacter sp*. (GQ390225)	12.2	Lake water
Proteobacteria;Gammaproteobacteria; Dasanias	uncultured gamma proteobacterium (GU061297)	3.4	Seawater at 20 m depth
Proteobacteria;Gammaproteobacteria; Granulosicoccus	uncultured bacterium (EU491601)	22.8	Seafloor lavas
Proteobacteria;Gammaproteobacteria; Lamprocystis	uncultured *Chromatiaceae* bacterium (AB478670)	3.2	Biofilm in wetland
Proteobacteria; Deltaproteobacteria; Desulfopila	uncultured bacterium (GU118230)	3.4	Corals (*Diploria strigosa*)
Proteobacteria; Deltaproteobacteria; Desulfuromusa	uncultured bacterium (GU291345)	22.1	Marsh sediments

Proteobacteria; Deltaproteobacteria; Desulfocapsa	uncultured bacterium (AM490747)	2.7	Cave sediments
Proteobacteria; Epsilonproteobacteria; Sulfurimonas	uncultured epsilon proteobacterium (AB189337)	6.6	Cold seep sediment
Proteobacteria; Alphaproteobacteria; Ahrensia	uncultured bacterium (FJ416098)	7.6	Sediment sea
Proteobacteria; Alphaproteobacteria; Anderseniella	uncultured alpha proteobacterium (GQ472791)	3.4	Surface water sea
Proteobacteria; Alphaproteobacteria; Caedibacter	uncultured bacterium (AY549548)	23.4	Endosymbiont of *Acanthamoeba* sp.
Proteobacteria; Alphaproteobacteria; Candidatus Captivus	uncultured bacterium (GQ389068)	30.1	Drinking water
Proteobacteria; Alphaproteobacteria; Candidatus Midichloria	uncultured bacterium (GU119441)	3.5	Reef water
Proteobacteria; Alphaproteobacteria; Cohaesibacter	uncultured bacterium (FJ792130)	3.4	Hydrothermal field
Proteobacteria; Alphaproteobacteria; Donghicola	uncultured bacterium (FJ792001)	2.3	Hydrothermal field
Proteobacteria; Alphaproteobacteria; Holospora	uncultured alpha proteobacterium (FM253609)	26.3	Rock biofilm
Proteobacteria; Alphaproteobacteria; Leisingera	uncultured alpha proteobacterium (EU050748)	3.8	Artic sediment
Proteobacteria; Alphaproteobacteria; Marinovum	uncultured *Rhodobacteraceae* bacterium (FJ403088)	3.5	Coral (*Montastraea annularis*)
Proteobacteria; Alphaproteobacteria; Maritimibacter	uncultured bacterium (FJ973591)	3.8	Water column
Proteobacteria; Alphaproteobacteria; Meganema	uncultured bacterium (EU835470)	18.9	Reverse osmosis membrane biofilm
Proteobacteria; Alphaproteobacteria; Methylarcula	*Methylarcula terricola* (AF030436)	3.4	Coastal saline
Proteobacteria; Alphaproteobacteria; Pelagibius	uncultured bacterium (EF125412)	2	Mangrove sediment
Proteobacteria; Betaproteobacteria; Albidiferax	uncultured prokaryote (GU208444)	29	Lake sediment
Proteobacteria; Betaproteobacteria; Conchiformibius	uncultured bacterium (GQ114730)	2.7	Human skin
Proteobacteria; Betaproteobacteria; Limnohabitans	uncultured bacterium (GU169064)	6.1	River water
Proteobacteria; Betaproteobacteria; Undibacterium	uncultured bacterium (AB199568)	25.5	River water
Proteobacteria; Betaproteobacteria; Vitreoscilla	uncultured bacterium (EU104016)	2.8	Activated sludge
Proteobacteria unclassified	uncultured bacterium (FJ628270)	5.7	Brackish water lake
Firmicutes; Clostridia; Anaerofustis	uncultured *Clostridiales* bacterium (AB198562)	3.4	Termite gut wall
Firmicutes; Clostridia; Butyrivibrio	uncultured bacterium (FJ681365)	26.6	Fecal beef cattle
Firmicutes; Clostridia; Gelria	uncultured bacterium (CU922123)	32.2	Wastewater sludge

Firmicutes; Clostridia; Helcococcus	uncultured bacterium (GQ014249)	3.1	Human skin
Firmicutes; Clostridia; Papillibacter	uncultured bacterium (FJ172870)	25.5	Rumen
Firmicutes; Clostridia; Peptoniphilus	uncultured bacterium (GQ072546)	3.1	Human skin
Firmicutes; Clostridia; Ruminococcus	uncultured bacterium (GQ046054)	2.1	Human skin
Firmicutes; Clostridia; Subdoligranulum	uncultured bacterium (GQ871735)	9.9	Cecum mucosa geese
Firmicutes; Clostridia; Anaerococcus	uncultured *Anaerococcus sp*. (DQ130021)	2.6	Human skin
Firmicutes; Clostridia; Dorea	uncultured bacterium (EU468966)	8.4	Gazelle feces
Firmicutes; Clostridia; Faecalibacterium	uncultured bacterium (DQ823804)	31.9	Human feces
Firmicutes; Clostridia; Fastidiosipila	uncultured low G+C Gram-positive bacterium SHD-209 (AJ278163)	2.8	River sediment
Firmicutes; Clostridia; Johnsonella	uncultured bacterium (GQ006796)	3.6	Human skin
Firmicutes; Clostridia; Anaerotruncus	uncultured rumen bacterium (GU304474)	2.8	Rumen epithelium
Firmicutes; Erysipelotrichi; Erysipelothrix	bacterium enrichment culture clone *DPF18* (GQ377127)	28.2	Duck waste
Firmicutes; Negativicutes; Succiniclasticum	uncultured rumen bacterium (AB185630)	2.5	Rumen
Firmicutes; Negativicutes; Acidaminococcus	uncultured rumen bacterium (GU303832)	3	Rumen
Firmicutes; Negativicutes; Anaerovibrio	uncultured bacterium (FJ189572)	3.7	Anaerobic sludge
Firmicutes; Bacilli; Gemella	uncultured bacterium (EU465711)	26.7	Sheep feces
Actinobacteria; Actinobacteridae; Actinospica	*Actinospica sp*. Gamma4 (AJ86586)	5.3	n.i
Actinobacteria; Actinobacteridae; Beutenbergia	*Beutenbergia cavernae* (Y18378)	24	Cave (L-lysine producer)
Actinobacteria; Actinobacteridae; Candidatus Aquiluna	uncultured bacterium (HM129136)	30.2	Water lake
Actinobacteria; Actinobacteridae; Collinsella	uncultured bacterium (DQ823740)	3.2	Human feces
Actinobacteria; Actinobacteridae; Fodinibacter	marine actinobacterium YM25-85 (AB522645)	24.4	Hard coral
Actinobacteria; Actinobacteridae; Glaciibacter	*Glaciibacter superstes* (AB378302)	24.1	Ice wedge
Actinobacteria; Actinobacteridae; Micropruina	uncultured bacterium (CU918282)	29.1	Wastewater sludge
Actinobacteria; Actinobacteridae; Planobispora	*Planobispora rosea* (AB028654)	29.2	n.i.
Actinobacteria; Actinobacteridae; Renibacterium	*Renibacterium salmoninarum* (AF180950)	26.4	n.i
Actinobacteria; Actinobacteridae; Schumannella	uncultured *Microbacteriaceae* bacterium (FJ542864)	26.6	Gut redworm
Actinobacteria; Actinobacteridae; Serinicoccus	*Serinicoccus sp*. 0714S6-1 (EU603762)	6	Deep-sea sediments
Actinobacteria; Actinobacteridae; Skermania	*Skermania piniformis* (AY788090)	3.9	Activated sludge foam
Actinobacteria; Actinobacteridae; Streptoalloteichus	*Streptoalloteichus hindustanus* (D85497)	24.7	n.i
Actinobacteria; Actinobacteridae; Dermabacter	*Dermabacter hominis* (X91034)	2.7	Surface cheese
Acidobacteria; Bryobacter; Bryobacter	uncultured bacterium (FJ466197)	30.9	Volcanic deposit

Fusobacteria; Fusobacteriales; *Leptotrichia*	uncultured bacterium (GQ000835)	27.1	Human skin
Thermotogae; Thermotogales; Geotoga	uncultured *Thermotogae* bacterium (EU721761)	3.5	Oil well
Thermotogae; Thermotogales; Kosmotoga	uncultured bacterium (DQ080153)	3	Sediment
Synergistetes; Synergistia; Aminobacterium	uncultured bacterium (CU925441)	25.7	Wastewater sludge
candidate division WWE1; Candidatus Cloacamonas	uncultured bacterium (CU918853)	7.6	Wastewater sludge

3.4. Discussion

The advent of high throughput DNA sequencing has set the stage for a major leap forward in our knowledge on the diversity, structure and functions of bacterial communities in agricultural systems. While the functional traits of the vast majority of bacteria are still unknown, studies on culturable taxa suggest that various functions that are of relevance to plant growth promotion (such as nutrient cycling and disease suppression) are associated with particular taxa even the same metabolic activity can be shared among different taxa. For examples, many pseudomonads and enterobacteriales are able to produce siderophores, antibiotics, auxins, and other activities such nitrogen-fixing which is commonly attributed to Rhizobiales (Sinorhizobium, Bradyrhizobium, Rhizobium) (Barret et al. 2011; Hayat et al. 2010). While much work has focused on the interactions of plants with PGPR pseudomonads, it is becoming increasing apparent, that the diversity of the plant rhizosphere is much greater than initially suspected from the first 16S rRNA clone library methods that were used to characterize the composition of plant rhizosphere communities.

In this work, we used high throughput pyrosequencing to determine the structure (diversity, distribution, richness) of the bacterial communities that develop in the ryegrass rhizosphere for plants grown in two Chilean Andisols. The 16S rRNA gene sequences obtained with these methods mostly represented individual OTUs, suggesting that the actual species diversity numbers in the thousands such that community structures are still best described at higher taxon levels where species can be clustered into phylogenetic groups. At the phylum level, our results revealed generally similar rhizobacterial communities for ryegrass plants grown in both Andisols. The major phyla included Proteobacteria, Actinobacteria and Acidobacteria as dominant groups in both rhizosphere microsites. In contrast, two phyla that varied in abundance between the two

soils were Cyanobacteria, which occurred in higher numbers in the Freire series, and Firmicutes, which appeared to be enriched in the Piedras Negras series. The analysis of rhizosphere bacterial communities at the species level using VITCOMIC software also showed similar structures of the bacterial communities for the two rhizosphere microsites, with members of the Proteobacteria and Cyanobacteria common to both microsites. These data are in agreement with prior studies using molecular based methods, which have demonstrated that the rhizobacterial community is generally represented by Proteobacteria, Actinobacteria and Acidobacteria (Arjun and Harikrishnan 2011;de Campos et al. 2013). Similarly, based on *GenBank* database, Janssen (2006) concluded that the members of the Proteobacteria and Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes (Bacilli) and Verrucomicrobia phyla are the most abundant soil bacteria. Likewise, use of pyrosequencing methods by Yang et al. (2012) revealed that members of Actinobacteria, Proteobacteria, Acidobacteria, Gemmatimonadetes phyla were dominant in the rhizosphere of pea plants. Pyrosequencing techniques have also revealed that Acidobacteria, Proteobacteria, Actinobacteria were the most abundant groups in soil bacterial communities associated with native hardwood forest and conifer plantations (Lin et al. 2011). However, interpretation of 16S rRNA gene-derived results must be approached with caution when used to quantify the relative abundance of different taxa as 16S rRNA gene copy numbers per genome vary from 1 up to 15 or more copies depending of bacterial species could produce an overestimation of abundance values (Větrovský and Baldrian 2013). Also, we need to consider the soil DNA extraction process, particularly the extraction efficiency (DNA extracted versus total soil DNA) and the presence of extracellular DNA derived from dead cells in soil (Bastida et al. 2009; He et al. 2005; Lombard et al. 2011).

Our results also showed that Fusobacteria, Thermotogae, Lentisphaerae, Tenericutes, Deferribacteres Spirochaetes, Planctomycetes, Thermotogae, Deinococcus-Thermus were the least abundant phyla in the rhizosphere of *Lolium* with <1% relative average abundance. In this context, the numbers of rRNA gene copies were likely related to the growth conditions, e.g. bacterial taxa with low copy number of 16S rRNA gene have been assumed to inhabit low-nutrient environments (Větrovský and Baldrian 2013). Little is known about bacterial groups with low abundance and activity in soils. Most of these phyla have not been studied yet and their role in soils and rhizosphere remain unknown.

With respect to differences among soils, the only significant differences in community structures were for the relative populations sizes of Cyanobacteria and Firmicutes. This result could possibly be influenced by differences in agricultural practices. Several studies have shown that agricultural practices (rotation, tillage, fertilization, etc.) influence the structure and activity of microbial community in soils. PLFA and DGGE techniques revealed higher diversity and abundance of microbial populations present in pasture soils under monoculture, respect to soils under rotation (Garveba *et al*. 2004). Suzuki et al. (2009) also observed that bacterial communities are more dependent of soil type (Cumulic Andosol, Low-humic Andosol, Yellow Soil and Gray Lowland Soil) than fertilizer type (chemical fertilizer, rice husk and manure). Chaer et al. (2009) reported a decrease in the diversity and enzymatic activities of soil microorganisms in cropped corn (Zea mays) and pigeon pea (*Cajanus cajan*) subjected to long-term conventional tillage. In Chilean Andidols, Martínez *et al*. (2011) observed that N fertilization (urea) influenced the occurrence of rhizobacteria with plant growth promoting activities, particularly increasing the abundance of phosphobacteria and decreasing the abundance of auxin-producing bacteria.

In the present study, there were no significant differences in the structures of bacterial communities between rhizosphere microsites (root tips and mature zone). This contrasts with previous studies, which reported differences in bacterial community structures of rhizosphere microsites (root tip, elongation and mature zone) by using DGGE, TRFLP and microarray techniques, (DeAngelis et al. 2009; Jaeger et al. 1999; Marschner et al. 2001a and 2001b, Marschner et al. 2004). It is known that these techniques have lower resolution compared with 454 sequencing technique and they consider dominant bacterial fraction in their analysis without consider the low abundance bacterial populations, which is reached by pyrosequencing. On other hand, the absence of significant differences between microsites could due to differences in the strength of the rhizosphere effect among plant species. It has been reported that the influence of plant roots on the rhizosphere decreases with the distance from the root surface, and will also depend on the diameter of the roots, their biomass, and amount of carbon that is deposited into the rhizosphere. Many studies on the rhizosphere use coarse rooted plants such as bean and maize that have large diameter roots (1 mm) where it is relatively easy to collect soil from the rhizoplane and where large biomass leads to a greater radial diameter of the rhizosphere effect. In contrast, ryegrass has very fine roots (< 0.1 mm) and the extent of the rhizosphere influence is much smaller than with more coarse rooted plants (Hinsinger *et al*. 2005). With fine rooted plants, collection of true rhizosphere soil is more difficult than with coarse rooted plants. Ryegrass roots also have inherently fast extension rates such that the time in which the root exudates are deposited into a particular zone in the soil is much shorter. As modeled by Watt et al. (2006) these spatial-temporal factors can strongly influence the strength of the rhizosphere effect among different plant species.

It is noteworthy that pyrosequencing analysis revealed the occurrence of bacterial genera that have not been previously reported to occur in the rhizosphere environment according to the *GenBank* database. Nonetheless, the alignments with reference sequences present in SILVA database showed low percentage similarities suggesting the occurrence of rhizobacterial species which have not yet been registered in online databases. Thus, our study demonstrates that the microbial diversity in the ryegrass rhizosphere grown in Chilean Andisol is much greater than those estimated previously based on conventional molecular techniques. In traditional molecular studies, dominant populations have masked the detection of low abundance OTUs, genetic diversity, and their individual distribution in soil environments. Studies based on pyrosequencing analysis have recently uncovered relatively "rare" species in soil bacterial communities (Dini-Andreote and Elsas 2013). The distribution and activity of these bacterial groups in rhizosphere microsites as well as their role in soil nutrient cycling and plant growth remain unknown.

3.5. Conclusions

The present study showed that Proteobacteria, Actinobacteria, Acidobacteria are dominant phyla colonizing the rhizosphere of *Lolium* grown in Chilean Andisols. However, the dominant groups represent only 5-10% of total bacterial communities demonstrating that low-abundance bacterial groups are principal component of rhizobacterial community. At the phylum level, differences in the abundance of Cyanobacteria and Firmicutes were only observed in two Andisols used. Our results did not show differences in the structure of bacterial communities between rhizosphere microsites (root tips and mature zone) in the same Andisol series. Pyrosequencing analysis also revealed the occurrence of bacterial genera, which have not been previously described for soil or rhizosphere according to *GenBank*. This study shows in detail the rhizobacterial communities associated with *Lolium* grown in Chilean Andisols and revealed the high proportion of low-abundance of bacterial groups, including novel members do not described for soil and rhizosphere. This information is relevant to studies on microbial ecology in the rhizosphere and use of bacteria-based biofertilizers in agriculture.

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CHAPTER 4

Effect of phosphorus addition on total and alkaline phosphomonoesterase-harboring bacterial populations in ryegrass rhizosphere microsites

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Effect of phosphorus addition on total and alkaline phosphomonoesterase-harboring bacterial populations in ryegrass rhizosphere microsites

Abstract

Rhizobacterial communities may play a crucial role in phosphorus (P) nutrition of plants. However, our knowledge of how P fertilization modulates rhizobacterial communities in crops and pastures is still poor. Here we investigated the effect of P addition (phosphate [PHO] and phytate [PHY]) on the composition of total bacterial communities and alkaline phosphomonoesterases (APase)-harboring bacterial populations in the rhizosphere microsites (root tip [RT] and mature zone [MZ]) of *L. perenne*. Sizes and diversities of bacterial communities were studied by 454-pyrosequencing of 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR). Our results suggested that phosphorus addition induces significant changes in the rhizobacterial community composition. Despite that pyrosequence analysis showed that members of the *Proteobacteria*, *Actinobacteria*, *Chloroflexi* and Acidobacteria were the dominant phyla in all sampled rhizosphere microsites, differences in the relative abundances of some bacterial genera were detected (e.g., *Arthrobacter* and *Acidothermus*). Greater richness in rhizosphere microsites of plants supplied with PHY compared with PHO were revealed. With respect to APase-harboring bacterial populations, DGGE (*phoD* gene) showed significant differences between microsites supplied with PHO, PHY and controls. qPCR (16S rRNA genes, *phoD* and *phoX*) showed significantly greater abundances of bacteria and APase genes in RT than in MZ microsites. This study contributes to our

understanding of the effect P fertilization on rhizobacterial community compositions of pastures grown in Chilean Andisols.

Keywords: bacterial community; alkaline phosphomonoesterases; phosphorus fertilization; rhizosphere; ryegrass

4.1 Introduction

The composition of rhizobacterial communities differs along the length of roots due to selective enrichment of different populations according to the amount and composition of root exudates, resulting in a heterogeneous distribution of rhizobacterial communities (Marschner et al. 2011). For example, in the root tips zone, exudation rates are high and colonizers use easily metabolizable sugars and organic acids. This is in contrast to mature root zones, where the bacterial growth is lower due to lower input of metabolizable carbon (C) (Lagos et al. 2015; Marschner et al. 2011). Hence, this might alleviate a potential competition for essential nutrients acquisition between roots and microbes. Consequently, to examine how microorganisms and plants interact spatially and temporally along roots for nutrient acquisition may contribute to a better understanding of the mechanism involved and for development of cultivars that are efficient in acquisition of limiting nutrients, such as phosphorus (Spohn and Kuzyakov 2013).

Phosphorus (P) is an essential macronutrient and is frequently abundant in soils. However, bioavailability of P may be limited in soil for a variety of reasons. P is present in organic (Po) and inorganic (Pi) forms. Nevertheless, only inorganic orthophosphate anions (PO_4^{3-}) in soil solution are utilized by plants. In rhizosphere soils under P deficiency, some bacteria have the capacity to mineralize/solubilize Po and Pi (Richardson 2001). These processes have special importance in the rhizosphere where microbial activity may result in an increase in P availability to plants, while plants reciprocate by supplying C to bacteria (Marschner et al. 2011). Several studies have shown that addition of P modulated the bacterial composition in rhizospheres (DeForest and Scott 2010; Jorquera et al. 2013, 2014; Unno and Shinano 2013). Based on culture-dependent studies, Mander et al. (2012) demonstrated that low P status induces a

selective pressure in the environment that may lead to a change in the bacterial taxa composition that is associated with fitness-based species selection. Thus, species composition and diversity indexes along roots are two functional indicators of the bacterial colonization regulated by P status and their role in P cycling.

Microorganisms can access and recycle P by reducing pH via production of organic acids, e.g., citrate, malate, oxalate and gluconate (Marschner et al. 2011; Miller et al. 2010; Uroz et al. 2010), and releasing enzymes such as phosphatase (phosphomonoesterases and phosphodiesterases), which hydrolyze the orthophosphate group from organic compounds (Nannipieri et al. 2011). Alkaline phosphomonoesterases (APase) are encoded by different genes, including *phoD* (Gomez and Ingram 1995) *phoX* (Wu et al. 2007), and *phoA* (Ray et al. 1991). Among these, *phoD* has been widely studied as a functional marker for monitoring the distribution and diversity of APase harboring bacterial populations in soils (Fraser et al. 2015a, 2015b; Tan et al. 2013). Chhabra et al. (2013) and Fraser et al. (2015a) proposed that the diversity and abundance of genes encoding APase in alkaline/acid soils are affected by management practices such as P fertilization. Spohn and Kuzyakov (2013) showed that Po mineralization along roots differs according to Pi fertilization and that rhizodeposition and APase harboring microorganisms were not directly related.

Volcanic ash-derived soils (Andisols) are important for the economy of southern Chile, supporting the bulk of agricultural and forestry production. The major characteristics of Chilean Andisols are high contents Po (mostly phosphomonoesters), but with low P availability to plants, and high content of organic matter (Borie and Rubio 2003; Velásquez et al. 2016). In addition, about 40% of Chilean Andisols contains ryegrass (*Lolium perenne*) which is used for beef cattle and dairy milk in Southern Chile (Mora et al. 1999, 2004). Therefore, the application of large amounts of

P fertilizers are needed to maintain available P levels in soils and consequently ryegrass yields. However, our knowledge of P fertilization effects on nutrient cycling, availability, and its influence on the ecology of rhizobacterial community composition in Chilean Andisol is still poor. Thus, the main goal of this study was to assess effects of P sources on rhizobacterial composition along the roots of ryegrass (*Lolium perenne*) and its effect on abundance of two genes encoding APase (*phoX* and *phoD*).

4.2 Materials and Methods

4.2.1 Soil sampling and chemical properties

Soil samples of Andisol, Piedras Negras series, were collected from a ryegrass pasture located in the Los Ríos region (40°20'S, 72°35'W) and transported immediately on ice to laboratory. Piedras Negras series was chosen because it is a P-deficient soil that has been used as a model soil by our research group (Martinez et al. 2015; Menezes-Blackburn et al. 2014; Paredes et al. 2011). Chemical analyses of soil were conducted as follows: Inorganic N extracted with 2 M KCl and NO_3^--N was determined by the Devarda alloy distillation method (Radojević and Bashkin 1999). Available P (P_{Olsen}) was extracted using 0.5 M Na-bicarbonate and analyzed using the molybdate method (Murphy and Riley 1962). Organic matter contents were estimated by wet digestion (Walkley and Black 1934). Soil pH was measured in 1:2.5 soil/deionized water suspensions. Exchangeable potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+) were extracted with 1M ammonium acetate (CH_3COONH_4) at pH 7.0 and analyzed by flame atomic adsorption spectrophotometry (FAAS) (Warncke and Brown 1998). Exchangeable aluminum (Al^{3+}) was extracted with 1M KCl and analyzed by FAAS (Bertsch and Bloom 1996). In addition, soil samples were named as bulk soil (BULK) and also subjected to molecular analysis as described below.

4.2.2 Experimental design

Root boxes were built according to Lagos et al. (2014), with a triplicate complete randomized design in rhizotrons for each treatment. The ryegrass (*Lolium perenne* var. Nui) plants were introduced by planting pre-sterilized seeds (treatment for 3 min in a solution of 5% v/v sodium hypochlorite and rinsed with six changes of sterile demineralized water for 3 min). Ten seeds were planted per rhizotron at a depth of 2 cm and were placed in a growth chamber with the same conditions as described Lagos et al. (2014). Phosphorus was applied as potassium phosphate (KH_2PO_4, Merck, Darmstadt, Germany) at a rate of 0.3 g P kg^{-1} of soil (PHO), and a phytate solution ($C_6H_{18}O_{24}P_6$ and H_2O, Sigma-Aldrich Co., USA) at 0.825 g phytate kg^{-1} of soil (PHY). Concentrations of both chemicals were selected according to Jorquera et al. (2013) and Paredes et al. (2011). Later, soil supplemented with P sources were homogenized, moistened to field capacity (60%), incubated in plastic bags for 7 days at 20°C, and then used for experiment with root boxes. Plants grown without P addition were included as controls (CT). Bacterial community response was evaluated through short-term assays during 30 days under greenhouse conditions (20°C, ~60% of humidity and 8:16 h light: dark cycle).

4.2.3 Rhizosphere microsite sampling

Samples from microsites were taken according to described by Lagos et al (2014). Firstly, plants were carefully removed from the rhizotrons and root pieces with adhering soil were collected from the microsites of root tips (RT) and mature zones (MZ), according to considerations of Marschner et al. (2001) and DeAngelis et al. (2009). The root pieces (0.5~1 cm of length) were obtained from five or six plants for each rhizotron in triplicate. Then, soil aggregates were removed from root pieces by vortexing and

collected in a sterile polypropylene microtube per each rhizotron. The total DNA extraction from soil microsites samples was done as follow.

4.2.4 Soil DNA extraction

For DNA extraction, each rhizosphere sample (1 to 2 g) was pretreated with phosphate buffer according to Ogram et al. (1987). Total DNA was extracted from rhizosphere samples using a MoBio Power Soil® DNA Isolation Kit and MoBio Powerlyzer® homogenizer (MoBio Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions. DNA quality and quantity were determined by spectrophotometry (Thermo Scientific Multiskan GO UV/Vis model). The DNA purity was assessed by determination of A260/A280 absorbance ratio ~1.8 and DNA samples were used for molecular analysis (pyrosequencing, DGGE and qPCR).

4.2.5 454-Pyrosequencing analysis

First, the bacterial community composition in the rhizosphere and bulk soil samples were determined by 454-pyrosequencing analysis. Libraries of 16S rRNA genes were built per treatment and sequenced by Macrogen, Inc. (Seoul, Korea) using a Roche 454 GS-FLX Titanium Sequencer. The genomic DNA was amplified separately by PCR reaction using the bacterial primer pair UNI_AMP-27F (5'-AxxxG AGT TTG ATC MTG GCT CAG-3') and UNI_AMP-518R (5'-BWT TAC CGC GGC TGC TGG-3'), where A and B represent primers (CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG and CCT ATC CCC TGTG TGC CTT GGC AGT CTC AG) and xxx represent the barcode. The PCR conditions were: a hot-start at 95ºC for 3 min, and then 35 cycles at 94ºC for 15 s, 55ºC for 45 s, and 72ºC for 1 min. A final extension step was carried out at 72ºC for 8 min.

The sequence data were processed by MOTHUR version 1.34.0 (http://www.mothur.org; Schloss et al. 2009). The filtering and demultiplexing,

reduction of sequence error, filtering of low-quality reads, trimming of the barcode sequences, and alignment and detection of chimeras with UCHIME (Edgar et al. 2011) were carried out according to standard operational procedures (http://www.mothur.org/wiki/454_SOP; Lagos et al. 2014). After filtering, sequences were clustered into species-level operational taxonomic units (OTUs) at 3% dissimilarity cutoff. Each OTU was assigned to the species level, using a naive Bayesian classifier provided by the SILVA 16S rRNA database at a 0.8 confidence threshold. The sequences were aligned against those sequences deposited in the SILVA database using the kmer search tool to find template sequences and Needleman-Wunsch algorithm to align them. Sequences exhibiting at least 95% sequence similarity over at least 302 bp were assigned to each species.

Alpha and Beta diversity patterns were calculated in MOTHUR. Alpha-diversity indexes were estimated based on the observed number of OTUs, and, for each soil microsite sample, relative abundances were determined. The relative abundance of OTUs that were differentially distributed among populations was statistically evaluated using Metastats in MOTHUR. Chao1 and inverse Simpson (1/D) indexes were calculated, and rarefaction curves were used to assess species richness. For beta-diversity, Jaccard, Yue and Clayton theta and Morisita Horn similarity indexes were calculated, and the diversity estimators were visualized using a heat map. Nonmetric multidimensional scaling (NMDS) plots and cluster analysis derived from Bray Curtis distances between bacterial communities of rhizosphere microsites were obtained.

4.2.6 Analysis of APase-harboring bacterial populations

Bacterial populations harboring APase (*phoD*) genes were evaluated using DGGE as described by Jorquera et al. (2014) and Sakurai et al. (2008). The DNA from microsites samples was performed in triplicate. The *phoD* gene fragments were amplified by PCR

using primer set ALPS-F730/ALPS-R1101-GC (Table 4.1). The PCR conditions were: a hot start of 3 min at 94ºC, and then 35 cycles of 94ºC for 1 min, 57ºC for 1 min and 72ºC for 2 min followed by final extension step of 7 min at 72ºC. The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories Inc., USA). Twenty microliter aliquots of PCR products were loaded onto 6 % (w/v) polyacrylamide gel with a 50 to 75% denaturing gradient (7M urea and 40% formamide) and the electrophoresis was run for 12 h at 100 V. The gel was stained with SYBR Gold (Invitrogen^{TM}, Thermo Fisher Scientific Inc., USA) for 30 min and photographed with GelDoc-It^{TS2} (UVP, USA).

Analysis of APase-harboring bacterial populations and quantification of dominant bands was carried out using Phoretix 1D Pro Gel Analysis Software (TotalLab Ltd., UK), using an unweighted pair group method with arithmetic averages (UPGMA) to build the dendrograms. Based on the matrix obtained from Phoretix analysis, the changes in the bacterial community composition between soil samples were calculated by similarity profile analysis (SIMPROF test) with Bray-Curtis similarity index, 5% significance level and <0.1 stress values (Clarke 1993; Clarke et al. 2008), and visualized by non-metric multidimensional scaling (NMDS) analysis using Primer 6 software (Primer-E Ltd., UK). Bacterial diversity by richness (S), Shannon-Wiener (H) and Simpsons (1/D) indexes was also calculated (Haegeman et al. 2014; Yang et al. 2003).

4.2.7 APase genes abundance analysis

The abundances of APase genes (*phoD* and *phoX*) were estimated by quantitative PCR (qPCR) using the primer set phoX2-F/phoX2-R and ALPS-F730/ALPS-R1101 (Table 4.1) as described by Acuña et al. (2016) using Applied Biosystems 7300 Real Time PCR System (Thermo Fisher Scientific Inc., USA). The DNA from microsites samples

was performed in triplicate with 3 technical replicates. All PCR reactions were performed in using Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences, UK) following manufacturer´s instructions. The primer sets used and qPCR conditions were: 1) for *phoD*: a hot start at 95 °C for 10 min, and then 40 cycles at 95°C for 15 s, 60 °C for 1 min, followed at 95°C for 15 s, 60°C for 1 min, and final extension step at 95°C for 15 s, and 2) for *phoX*: a hot start at 95 °C for 10 min, and then 40 cycles at 95°C for 15 s, 60 °C for 1 min, followed at 95°C for 15 s, 60°C for 1 min, and final extension step at 95°C for 15 s. The universal primer set targeting 16S rRNA gene, 1055-F/1392-R (Table 4.1), was used to quantify the APase genes in relation to the total bacterial DNA. The qPCR conditions were: a hot start at 95°C for 30 s, and then 35 cycles at 95°C for 30 s, 50°C for 30 s, 75°C for 30 s, followed at 95°C for 15 s, 60°C for 1 min, and final extension step at 95°C for 15 s. The standard and dissociation curves were constructed to evaluate the efficiency and specificity as described by Acuña et al. (2016). The normality of the data was calculated by Shapiro–Wilks' test, and then analyzed by two-way analyses of variance (ANOVA) and Tukey as post-hoc test with SPSS software (IBM SPSS Inc., USA). Differences were considered significant when the *P* value was less than or equal to 0.05.

Table 4.1. Primer sets used in this study

Genes target	Primer name	Sequences (5'–3')	References
qPCR			
16S rRNA	1055-F	ATG GCT GTC GTC AGCT	Harms *et al.*, 2003
	1392-R	ACG GGC GGT GTG TAC	
phoD	ALPS-F730	CAG TGG GAC GAC CAC GAG GT	Sakurai *et al*., 2008
	ALPS-R1101	GAG GCC GAT CGG CAT GTC G	
phoX	phoX2-F	GAR GAG AAC WTC CAC GGY TA	
	phoX2-R	GAT CTC GAT GAT RTG RCC RAA G	Sebastian and Ammerman 2009
DGGE			
16S rRNA	EUBf 933-GC[a]	GCA CAA GCG GTG GAG CAT GTG G	Iwamoto *et al*., 2000
	EUBr 1387	GCC CGG GAA CGT ATT CAC CG	
phoD	ALPS-F730	CAG TGG GAC GAC CAC GAG GT	Sakurai *et al*.,2008
	ALPS-R1101-GC[b]	GAG GCC GAT CGG CAT GTCG	

[a]GC- clamp, CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG. [b]GC- clamp, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G. All GC-clamps were attached to the 5'-end of primer

4.3 Results

4.3.1 Soil chemical properties

The chemical properties of soils are presented in Table 4.2. Differences were observed between rhizosphere soils (PHO, PHY and CT) and collected soil samples (BULK). The available P was significantly greater ($P \leq 0.05$) in soil supplemented PHO (31 mg kg^{-1}), followed by PHY (15 mg kg^{-1}), CT (7 mg kg^{-1}) and BULK (6 mg kg^{-1}) soils. The pH values were lower in rhizosphere soils (ranging from 5.36 to 5.47) than BULK soil (5.72). In contrast, significantly greater ($P \leq 0.05$) available N was observed in rhizosphere soils (ranging from 137 to 343 mg kg^{-1}) compared with BULK soil (21 mg kg^{-1}). Similarly, significantly greater ($P \leq 0.05$) cation exchange capacity (CEC) and Al saturation was greater in rhizosphere soils (3.23~6.29 $cmol_{(+)}$ kg^{-1} and 4.57~10.53%, respectively) than BULK soil (1.94 $cmol_{(+)}$ kg^{-1} and 2.58%, respectively).

Table 4.2. Average values (n=3) for some chemical properties of soil samples used in this study

		Rhizosphere soils		
	Bulk	PHY	PHO	CT
pH H_2o	5.72	5.47	5.38	5.36
Organic matter (%)	12	22	20	21
N (mg kg^{-1})	21	343	137	303
P (mg kg^{-1})	6	15	31	7
K (mg kg^{-1})	51	145	383	293
K ($cmol_{(+)}$ kg^{-1})	0.13	0.37	0.98	0.75
Na ($cmol_{(+)}$ kg^{-1})	0.07	0.21	0.19	0.15
Ca ($cmol_{(+)}$ kg^{-1})	1.45	4.03	3.91	1.64
Mg ($cmol_{(+)}$ kg^{-1})	0.24	0.82	0.77	0.35
Al ($cmol_{(+)}$ kg^{-1})	0.05	0.26	0.44	0.34
CEC ($cmol_{(+)}$ kg^{-1})	1.94	5.69	6.29	3.23
Al saturation (%)[a]	2.58	4.57	7	10.53

CT, soil without P supplementation; PHO, soil supplemented with phosphate; PHY, soil supplemented with phytate; BULK, bulk soil. [a]Calculated as (Al×100) / CEC, where CEC= cation exchange capacity=Σ (K, Ca, Mg, Na, and Al)

4.3.2 Analysis of bacterial community composition

A total 118,226 sequences of 16S rRNA gene fragments were obtained and used to determine the phylogenetic affiliation in each soil sample, and sequences were clustered into OTUs based on a distance of 0.03 cutoff (Table 4.3). In general, our results did not show significant difference ($P \leq 0.05$; Tukey's test) in the relative abundance (RA) between treatments and microsites at phyla level. The dominant phyla in rhizosphere samples were *Proteobacteria* (ranging from 30 to 48%). Within the *Proteobacteria*, members of the class *Alphaproteobacteria* were the most dominant (ranging from 18 to 28%), *Betaproteobacteria* (ranging from 9 to 17%) followed by *Gammaproteobacteria* (ranging from 1 to 5%) (Fig 4.1b). *Actinobacteria* (ranging from 13 to 26%), *Chloroflexi* (ranging from 7 to 18%) and *Acidobacteria* (ranging from 1 to 14%), independently of microsites (MZ and RT). In contrast, collected soil samples (BULK) showed members belonging to *Proteobacteria* (76%) and *Firmicutes* (15%) as dominant phyla (Fig. 4.1a). In relation to classes, members of the class *Betaproteobacteria* (44%) and *Gammaproteobacteria* (25%) were the most dominant (Fig.4.1b). In RT microsites, the RA of *Chloroflexi* and *Acidobacteria* decreased when rhizosphere soils were supplemented with PHO (7% and 3%, respectively) compared with PHY (14% and 10%, respectively) and CT (16% and 11%, respectively). In contrast, the RA of *Bacteroidetes* increased in rhizosphere soils supplemented with PHO (10%) compared with with PHY (1.5%) and CT (1.8%, respectively). In relation to MZ microsites, greater RA of *Acidobacteria* were only observed in rhizosphere soils supplemented with PHO (14%) and PHY (12%) in comparison with CT (1.5%). It is noteworthy that abundances are expressed in relation to total community; some caution is therefore required not to over-interpret the results. For example, if the abundance of one taxon is

higher under a certain condition, then the abundance of other taxa must necessarily be lower.

A total of 316 sequences were identified at the genus level. In general, the genus *Arthrobacter* was the most abundant group across all rhizosphere soils (ranging from 3% to 22% of RA), followed by *Massilia* (from 2% to 18%). The dominant genera and their RA differed when contrasted with collected soil (BULK), where *Stenotrophomonas* (19%), *Massilia* (15%) and *Bacillus* (9.7%) were dominants. When comparingthe RA of OTUs in each P treatment versus CT, Metastats analysis showed significant differences ($P \leq 0.05$). In MZ microsites (Fig. 4.2a), the addition of PHO increased the RA of *Variovorax*, but decreased the RA of *Rhodoplanes*, *Pseudolabrys* and *Acidothernus*. When MZ microsites were supplied with PHY, *Arthrobacter*, *Flavisolibacter*, *Acidothermus* and *Burkholderia* increased their RA. In RT microsites (Fig. 4.2b), the RA of *Duganella*, *Streptomyces*, *Polaromonas*, *Arthrobacter* and *Pedobacter* increased when soils were supplied with PHO; however, the RA of *Acidothermus* and *Flavosolibacter* decreased. When PHY was added a decrease of *Sphingomonas* was observed. It is noteworthy that the RA of *Acidothermus* decreased at both microsites when PHO was added, and increased in MZ when PHY was added.

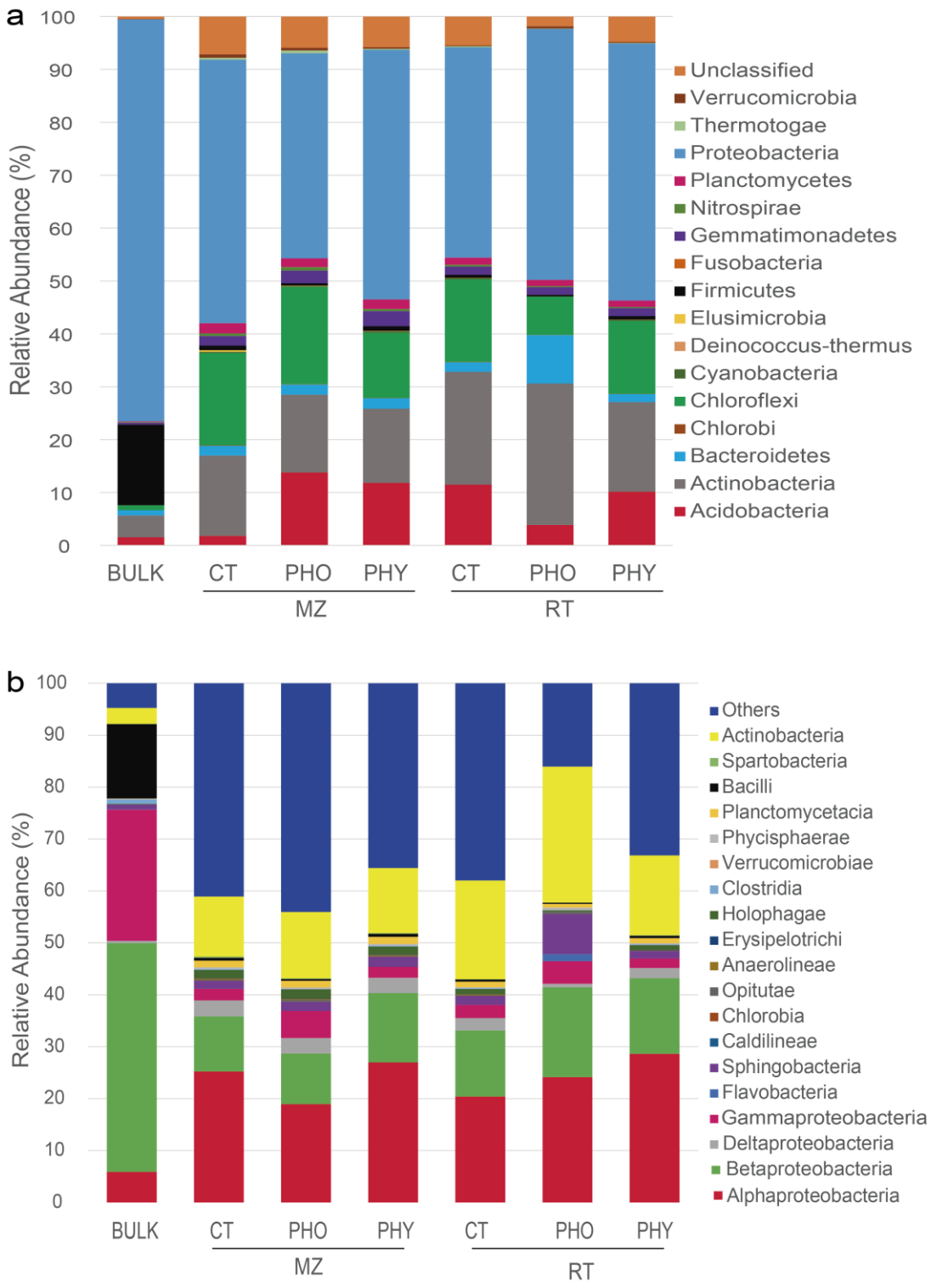

Figure 4.1. Relative abundance of phyla (**a**) and classes (**b**) in different rhizosphere microsites of ryegrass grown in soil supplied with different P sources. Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil

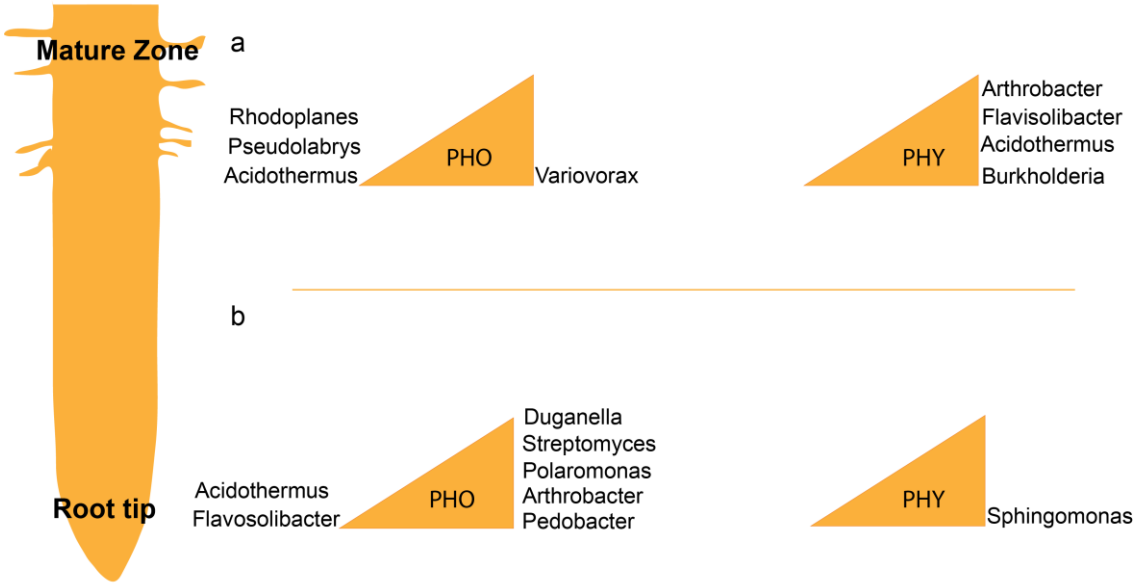

Figure 4.2. Analyzed by Metastats shown OTUs identified at genus level different statistically (*P* and *q* <0.05) which increased or decreased relative abundance according to P treatments in comparison with control treatment

In relation to alpha diversity analysis, the rarefaction curves showed an average of 3,700 OTUs, except RT_PHO and BULK, which plateaued at 2,616 and 2,417 OTUs, respectively (Fig. 4.3). In relation to the CHAO1 estimator (Table 4.3), the richness was greater in soils supplied with PHY (9.05 and 9.19 for RT and MZ microsites, respectively), followed by CT (7.87 in both microsites) and PHO (5.80 and 6.9 for RT and MZ microsites, respectively). Based on Simpson index (1/D), the soil samples from root tip and supplied with PHO showed lowest richness (94.6) compared to the richness from other microsites samples (ranging from 134.3 to 209.7) (Table 4.3).

The Beta-diversity analysis visualized as heat map displayed similarity values for microsites samples. The Jaccard similarity coefficient indicated a dissimilarity between the percentages of shared species at least 20% (Fig. 4.4a). Yue and Clayton theta and Morisita Horn similarity coefficients (Fig. 4.4b and 4.4c) showed high similarities

among bacterial communities. This result is confirmed by dendrogram with similarities of approximately 90% among bacterial communities of rhizosphere microsites (Fig. 4.4d).

Table 4.3. Total number of reads and diversity indexes obtained from molecular analysis

	Rhizosphere soils						
	PHY		PHO		CT		
	RT	MZ	RT	MZ	RT	MZ	BULK
Pyrosequencing (16S rRNA gene)							
Number of reads	18,551	20,841	14,820	16,705	15,884	15,376	16,049
No. rarified reads	14,820	14,820	14,820	14,820	14,820	14,820	14,820
Good´s Coverage	0.85	0.85	0.89	0.87	0.85	0.86	0.90
Observed OTUs	4,097	4,538	2,616	3,798	3,818	3,748	2,417
Chao1 richness index	9.05	9.19	5.80	6.90	7.87	7.87	6.70
Simpson (1/D) index	134.3	138.2	94.6	197.5	209.7	135.1	16.9
DGGE (phoD gene)							
Richness (S) index	7.29±0.13b	8.071±0.98bc	8.01±0.22bc	5.81±0.44ab	9.65±0.58c	6.4±0.10ab	4.41±0.01a
Shannon- Wiener (H`) index	1.85±0.06abc	2.18±0.15b	2.1±0.03ab	1.65±0.12ab	2.2±0.17b	1.83±0.06abc	1.37±0.03a
Simpson (1/D) index	1.42±0.01ab	1.35±0.02a	1.37±0.01a	1.45±0.03ab	1.37±0.03a	1.41±0.01ab	1.51±0.05b

CT, soil without P supplementation; PHO, soil supplemented with phosphate; PHY, soil supplemented with phytate; BULK, bulk soil. Different lowercase letters in the same row denote significant difference ($P \leq 0.05$; Tukey's test)

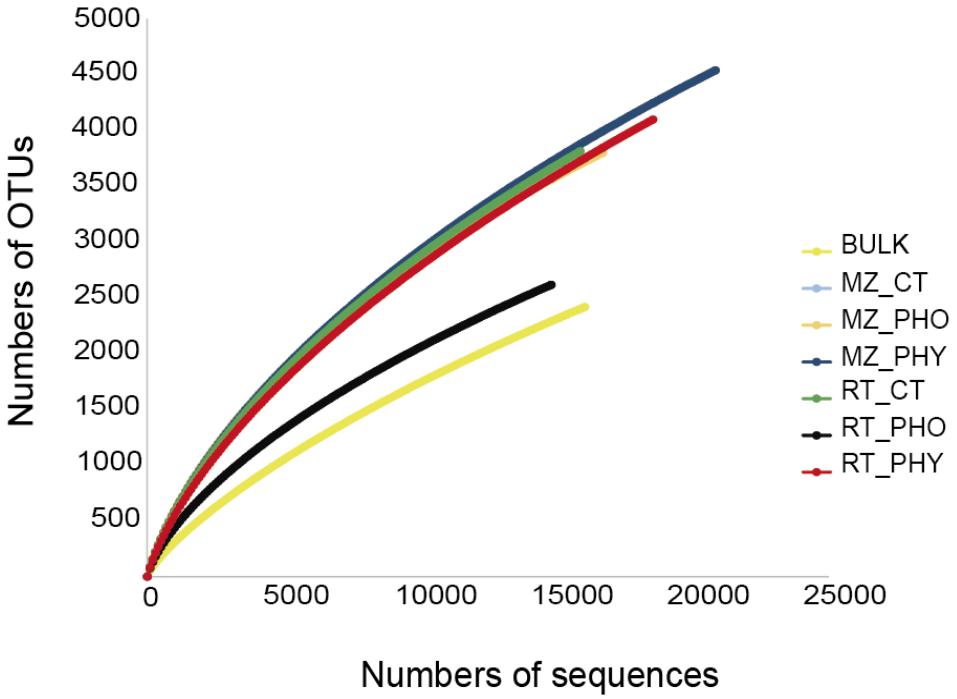

Figure 4.3. Rarefaction curves, showing the dependence of discovering novel OTUs as a function of number of sequences for OTUs defined at a 0.03 distance cut-off. Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil**.**

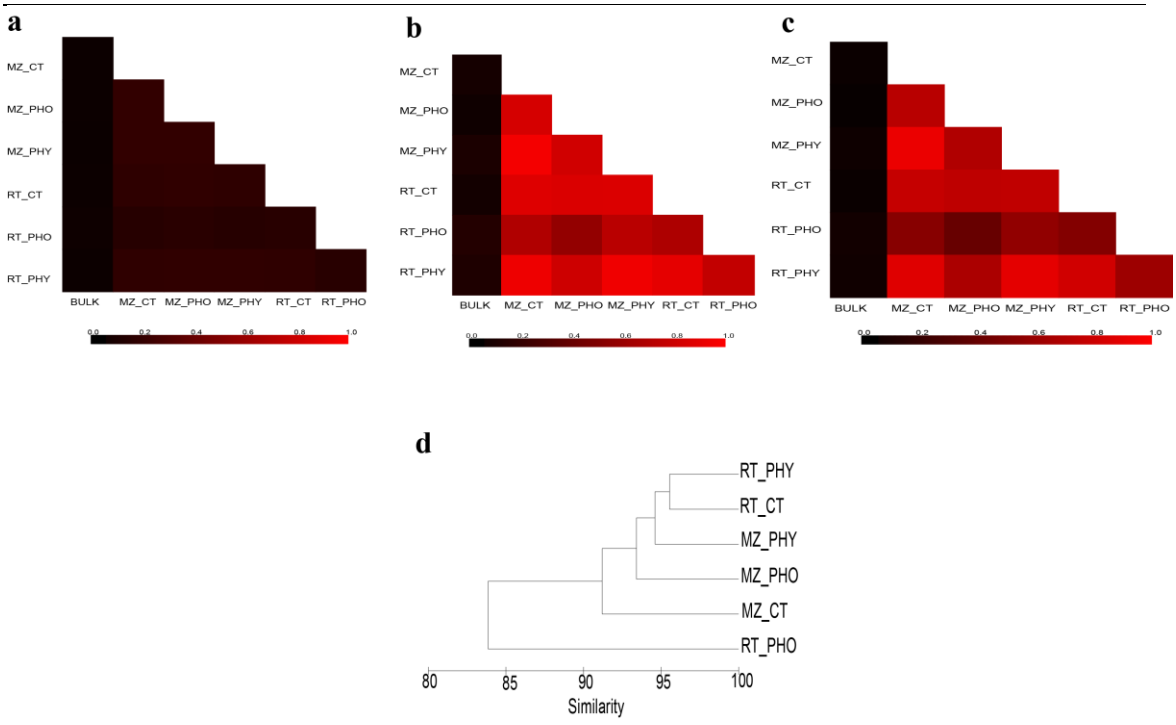

Figure 4.4. Visualization of Jaccard (**a**), Yue and Clayton theta (**b**) and Marisita Horn (**c**) similarity coefficients by heat map, OTUs defined at a 0.03 distance cut-off. Cluster analysis (**d**) derived from Bray Curtis distances at phylum level showing the similarity of bacterial communities of rhizosphere microsites. Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil.

4.3.3 Analysis of APase-harboring bacterial populations

At >40% similarity, the dendrogram from DGGE gels (*phoD*) grouped the APase-harboring bacterial population profiles according to P treatments (PHO and PHY) and CT, but not according to the rhizosphere microsites (MZ and RT) (Fig. 4.5a). This observation was confirmed by NMDS analysis (Fig. 4.5b), where bacterial community profiles were also grouped according to PHY, PHO and CT, but not according to sampled microsites (MZ and RT), except CT. With respect to bacterial diversity, the

Shannon-Weaver (H), richness (S) and Simpson (1/D) diversity indices did not show significant ($P \leq 0.05$) differences between treatments and microsites; however, in general the rhizosphere soils increased their diversity compared with collected soils (BULK) (Table 4.3).

4.3.4 Analysis of APase gene abundance

In general, our results showed significant ($P \leq 0.05$) differences in a greater copy numbers of 16S rRNA and APase genes (*phoD* and *phoX*) in RT than MZ microsites (Table 4.4). In relation to *phoX* genes, a greater copy numbers and RA of *phoD* genes were also found. Soil supplied with PHY showed greater gene copy number of *phoD* in RT (~8×10^{8}), whereas soil supplied with PHO showed greater gene copy numbers of *phoD* in RT (~5×10^{7}). In relation to the RA, *phoD* ranged from 2.87×10^{-4} to 13.6×10^{-4}, whereas *phoX* was ranged from 0.13×10^{-4} to 30.9×10^{-4}. However, significantly ($P \leq 0.05$) greater RA of both APase genes were observed in RT with PHO.

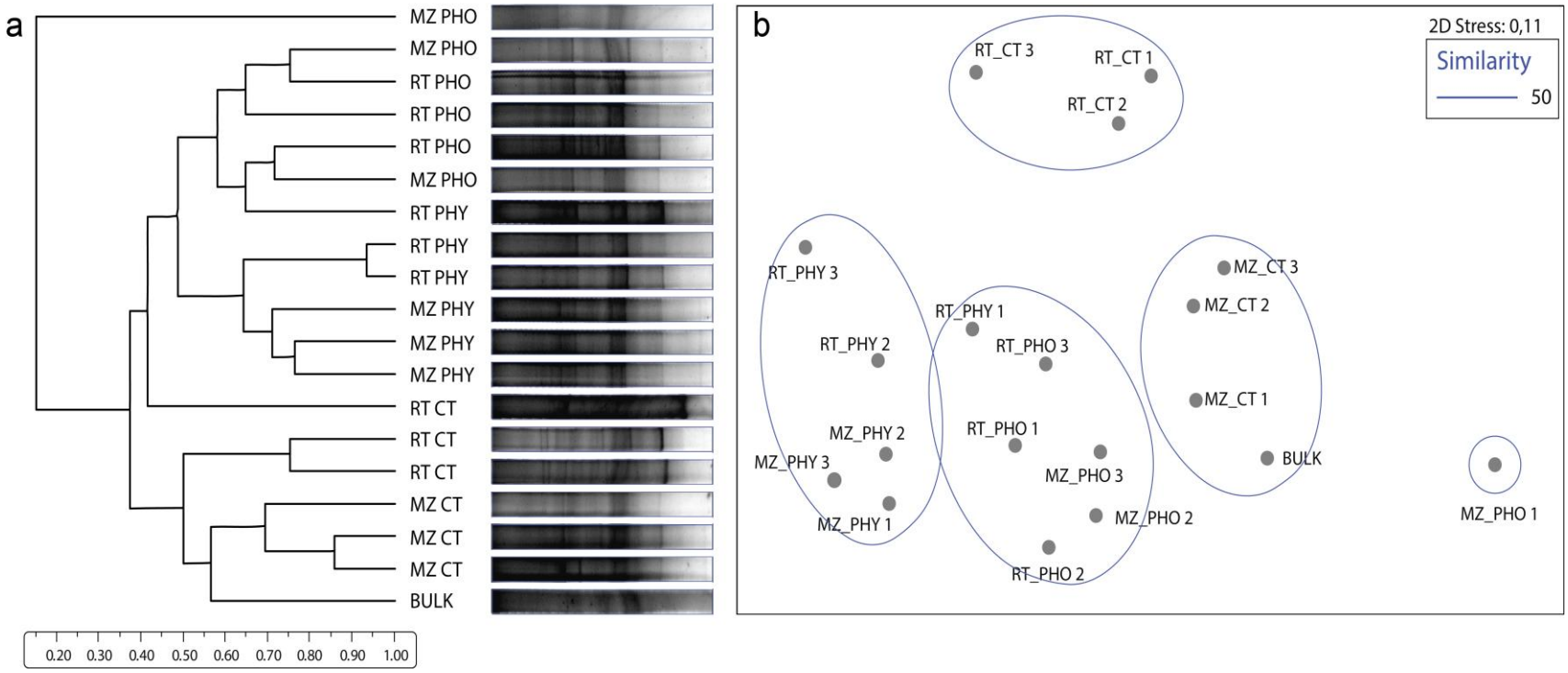

Figure 4.5. Dendrogram of DGGE profiles (**a**) and Nonmetric multidimensional scaling (NMDS) plots (**b**) derived from Bray Curtis distances of different rhizosphere microsites. Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil.

Table 4.4. Quantification of APase genes in soil samples by qPCR

Treatments		Absolute quantification (gene copy no.g^{-1} soil)			Relative quantification	
		16S rRNA ($\times10^{10}$)	*phoD* ($\times10^{6}$)	*phoX* ($\times10^{6}$)	*phoD* ($\times10^{-4}$)	*phoX* ($\times10^{-4}$)
PHY	RT	214±25.63f	794±100d	24.29±21.74d	4.05±0.61bc	0.13±0.02a
	MZ	1.06±0.05a	3.19±0.19a	1.44±0.96a	3.10±0.30b	1.35±0.29b
PHO	RT	1.79±0.02b	24.52±2.29b	54.55±5.78e	13.62±1.24d	30.90±3.86c
	MZ	19.46±1.90d	15.65±2.25ab	13.67±1.18c	0.94±0.23a	0.76±1.01b
CT	RT	70.74± 4.32e	199±14.76c	42.25±1.05de	2.87±0.23b	0.62±0.45b
	MZ	4.07±0.53c	14.17±2.86b	2.86±0.44b	3.22±0.40b	0.70±0.08b
BULK		4.49±0.18c	25.63±3.89b	3.88±0.38b	5.46±0.08c	0.75±0.03b

CT, soil without P supplementation; PHO, soil supplemented with phosphate; PHY, soil supplemented with phytate; BULK, bulk soil. Different lowercase letters in the same column denote significant difference ($P \leq 0.05$; Tukey's test)

4.4 Discussion

4.4.1 Bacterial community structure at root microsites

Changes in the rhizobacterial community composition in response to two P source applications were investigated in two the rhizosphere microsites of *L. perenne*. Pyrosequencing analysis did not show significant changes in bacterial community composition between microsites treated with PHO and PHY; however, significant changes were obtained when microsites of P fertilized plants were compared with BULK soils. Pyrosequencing also showed members of *Proteobacteria* (mainly *Alpha* and *Betaproteobacteria* classes) followed by *Actinobacteria*, *Chloroflexi* and *Acidobacteria* were dominant groups in all sampled microsites. Nevertheless, functional diversity is a common measure of bacterial community composition and may be more relevant to ecosystem function than taxonomic diversity. In this context, functional groups relevant to the rhizosphere including *Proteobacteria* as one of the most common phyla found to terrestrial ecosystems and characterized mainly by i) respond to labile sources of C and P, ii) have a fast rate of growth, and iii) adapt to diverse plant rhizospheres (Bulgarelli et al. 2013; Chaparro et al. 2014; Chhabra et al. 2013; Fraser et al. 2015a). Moreover, members of *Alpha* and *Betaproteobacteria* have mainly been associated with N cycling whereas *Gammaproteobacteria* have been related to a wider range of functions such as nutrient cycling (C and N), enteric, plant pathogens, among others (Hawkes et al. 2007). The *Acidobacteria* phylum have also been associated with the rhizosphere, increasing root nodulation and N mineralization in soils (Bulgarelli et al. 2013; Ward et al. 2009). At the genus level, our results showed that members of the *Arthrobacter* and *Massilia* were most abundant in all microsites samples. *Arthrobacter* is categorized among the top 20 most abundant bacteria in pasture soil (Acosta-Martinez

et al. 2008) and *Massilia* is considered a root colonizing bacteria of many plant species (Ofek et al. 2012).

At the genus level, Metastats analysis also revealed changes in bacterial communities induced by P addition into soil. Thus, greater and lower RA of specific bacterial genera were observed. For example, *Arthrobacter* shown a greater RA with both P sources in comparison to *Acidothermus* with a lower RA when PHO was applied. A decrease in RA of *Acidobacteria* and *Pseudomonas* has been attributed to Pi fertilization ($Ca[H_2PO_4]_2$) (Chhabra et al. 2013). Similarly, DGGE analysis shown changes of *Pseudomonas*, *Bradyrhizobium* and *Mesorhizobium* induced by P fertilization ($Ca[H_2PO_4]_2$ and H_2KO_4P) (Fraser et al. 2015a, 2015b; Jorquera et al. 2014; Pan et al. 2014; Tan et al. 2013). However, these bacterial groups differed than those revealed by Metastats analysis in our study. This may be due to the different molecular approaches used to assess bacterial community composition in rhizosphere microsites. It is recognized that the use of next generation sequencing technologies (such as 454-pyrosequencing and Illumina platforms) have become possible to characterize the composition and activity of bacteria populations in the environment at much greater resolution than before (Lagos et al. 2015).

Changes in bacterial community composition by P addition were also suggested by alpha diversity indexes, resulting in a greater richness in microsites supplied with PHY in comparison with those supplied with PHO. These results are agree with Unno and Shinano (2013) who reported a greater richness of bacterial groups in the rhizosphere of *Lotus japonicus* supplied with phytate compared with controls. Similarly, Wang et al. (2007) reported that microbial richness was greater when phytate was added compared to amended with Pi ($FePO_4$) and unamended soil. In this same context, studies on Chilean Andisols have demonstrated that phytate addition cause changes in bacterial

community composition in the rhizospheres of pasture, and induced the expression of phytase enzyme by an inoculated *Bacillus* MQH-19 strain (Jorquera et al. 2013). In contrast, long-term P fertilization did not produce significant changes in the composition of pasture rhizobacterial communities in field trial, compared with N fertilization (Jorquera et al. 2014). In the same study, the greater abundances of total bacteria were detected when greater doses of P and N fertilization (400 kg P ha^{-1} $year^{-1}$ plus 600 kg N ha^{-1} $year^{-1}$) were applied. In our study, the changes in bacterial community composition by P addition may be attributed to the ability of specific bacterial groups to mineralize and use phytate as a P and C source in the rhizosphere.

On the other hand, the beta diversity indexes did not show significant differences between bacterial community compositions produced by P fertilization. These results could be explained due to the data presented here come from a short-term experiment (30 days) after P supplementation and it is possible that greater differences could be detected with longer time of study (>8 years) of P fertilization as suggested by other investigators (Beauregard et al. 2010; Pan et al. 2014; Tan et al. 2013). Long-term fertilization also affects soil properties, such as pH, influencing the composition of bacterial populations in soils (Fierer and Jackson 2006; Lauber et al. 2009; Martinez et al. 2011; Rousk et al. 2010). Nevertheless, how each influenced soil property affects specific bacterial groups need also to be clarified (Hinsinger et al. 2003). In this sense, soil pH is considered one of major driver influencing the bacterial communities in the rhizosphere and bulk soils. The strong influence of pH in soil is due to changes produced in nutrients availability, releasing of toxic ions, changes in microbial respiration rates, among other (Hinsinger et al. 2003; Marschner et al. 2006). In fact, Lauber et al. (2009) reported that bacteria belonging to *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and Alpha, Beta and Gamma *Proteobacteria* were significantly

correlated with soil pH from 88 soils across North and South America. Similarly, Nacke et al. (2011) also concluded that these groups are correlated with pH in a grassland soil. In contrast, a recent study at global level reports predictable shifts in the taxonomy and functionality of soil microbial communities and those changes are most related with elevated nutrient (N and P) inputs that soil properties, such as soil pH (Leff et al. 2015).

4.4.2 APase-harboring bacterial populations at root microsites

In relation to analysis of APase-harboring bacterial populations, using *phoD* as target genes, the dendrogram and NMDS analysis performed by DGGE showed a clear separation between soil samples derived from PHO and PHY treatments and CT, but separation according to microsites was not observed. Recently, considerable changes in the composition and gene abundance of APase-harboring bacterial populations caused by application of organic and conventional fertilizers have been reported (Fraser et al. 2015a, 2015b; Tan et al. 2013). Based on PCR-DGGE (*phoD*), Fraser et al. (2015a) demonstrated that long term management may impact P cycling by differences in the abundance and diversity of *phoD* bacterial communities. Tan et al. (2013) assessed the effects of three fertilization rates in grassland soil and demonstrated changes in the bacterial community composition between control and treatments, being *Alpha* and *Gammaproteobateria*, *Actinobacteria* and *Cyanobacteria* dominants in P supplied soils. Similarity, Chhabra et al. (2013) demonstrated that APase-bacterial populations are more diverse in long-term P-fertilized soils than in soil without fertilization being *Alpha*, *Gammaproteobacteria* and *Cyanobacteria*. It is noteworthy that PCR primer set (ALPS) used to amplify APase genes may result in an over representation of *Alphaproteobacteria* class (Fraser et al. 2015b; Ragot et al. 2015); because ALPS primer set was mainly designed based on APases genes from *Alphaproteobacteria*

members and may not provide an adequate coverage of APases of bacterial world. Therefore, the interpretation and comparison of our results as well as the results of other studies must be viewed with some caution; they could represent only a portion of bacterial populations and not necessarily represent the total APase-harboring bacterial populations in each sample. Thus, new PCR primers are therefore required to improve the coverage of *phoD* gene diversity. In this context, Ragot et al. 2015 designed a new set of APase primer targeting *phoD* gene which amplified 13 phyla (e.g., *Verrumicrobia*, *Bacteroidetes*, *Chloroflexi*) in contrast with ALPS set primer which amplified 6 phyla. This study also showed that the structure of APase bacterial populations composition from 6 soil types with different content of P total (mg kg^{-1} soil) the bacterial groups more prevalent were *Alphaproteobacteria*, *Actinobacteria*, *Bacteriodetes*, *Chloroflexi, Cyanobacteria*.

The qPCR analysis showed greater abundance of APase genes (*phoD* and *phoX*) in RT than MZ microsites, with values ranging from 2×10^7 to 2×10^8 gene copy number. According to Acuña et al. (2016), the abundance of *phoD* in rhizosphere samples from plants of Chilean extreme environments ranged values between 6×10^4 and 9×10^8 gene copy number. Fraser et al. (2015a, 2015b) showed values of *phoD* abundance ranging between 3×10^6 and 1×10^7, in the rhizosphere of wheat grown in an agronomic soil with long term of organic manure management. In general, the RA of *phoD* was greater than *phoX* in our samples. Our results are agree with other authors who postulated that *phoD* is more abundant in terrestrial ecosystem than *phoX* and *phoA* (Luo et al. 2009; Tan et al. 2013). Nevertheless, recently, Acuña et al. (2016) revealed that the abundance the *phoX* gene was greater than *phoD* gene in some rhizosphere soils from native plant grown in Chilean extreme environments (Atacama Desert, Andes Mountains, Patagonia and Antartic), suggesting that *phoX* gene could be crucial for Po recycling in soil under

natural vegetation. Similarly, Morrison et al. (2016) revealed than the abundance *phoX* was greater than abundance *phoD* gene in four stations along a P gradient in soils of Everglades of Florida. The presence of bacterial APase genes has also been studied in aquatic ecosystems. These studies have reported greater abundance of *phoX* than *phoA* genes in seawater and fresh water bacteria (Sebastian and Ammerman 2009; Dai et al. 2014, 2015). In addition, Sebastian and Ammerman (2009) postulated that *phoX* gene is more widely distributed than *phoA* genes among marine bacteria. By metagenomic analysis, a greater relative abundance of *phoD* than *phoX* and *phoA* genes was also observed by Tan et al. (2013). The differences between abundances of APase genes in bacteria could be related with their subcellular localization. In fact, cytoplasmic APase encoded by *phoD* depend on internal cell P metabolism, whereas extracellular or periplasmic APase encoded by *phoX* genes depend on P uptake. The variation in the abundance of APase genes could obey to different ecological strategies for using organophosphate in P-depleted soil (Luo et al. 2009). As discussed before, our qPCR results by using ALPS primer set must be also viewed with some caution because they could represent only a portion of APase-harboring bacterial populations.

Summarizing, our results show that P fertilization can induce differences in the composition and abundance of total and APase-harboring bacterial populations between root microsites. As mentioned above, it has been postulated that bacteria are heterogeneously distributed and adapted along the plant roots according to the particular microsite condition, such as type and availability of nutrients (organic acids and sugars) released by plant roots (Marschner et al. 2011). In addition, molecular P transporters in plants are concentrated in some root regions, such root tip of lateral roots (Schachtman et al. 1998). Considering the type of P fertilization, the selection and/or persistence of specific bacterial populations (e.g., *Arthrobacter* with PHO and *Sphingomonas* with

PHY; Fig. 4.2b) and APase genes (Table 4.4 and Fig. 4.5) in specific rhizosphere microsites, such root tips, may play the most significant role in promoting plant P nutrition through the turnover of supplemented and/or fixed P forms in soils. However, further more studies are required to confirm this statement.

4.5 Conclusions

The present study suggests that P fertilization (phosphate [PHO] and phytate [PHY]) may induce changes in the composition of rhizobacterial communities of *L. perenne* grown in a Chilean Andisol. In general, changes in rhizobacterial community between soil supplied with PHO and PHY were not observed at phyla level. Pyrosequencing showed members of *Proteobacteria*, *Actinobacteria*, *Chloroflexi* and *Acidobacteria* as the dominant phyla in all sampled rhizosphere microsites. However, Metastats analysis revealed changes induced by P addition in the abundance relative of some bacterial genera, such as *Arthrobacter* and *Acidothermus*. Changes in bacterial communities were also suggested by alpha diversity indexes, resulting in a greater richness in microsites supplied with PHY compared with those supplied with PHO. In relation to alkaline phosphomonoesterases (APase)-harboring bacterial populations, using *phoD* as target genes, DGGE analysis showed significant differences between rhizosphere supplied with PHO, PHY, and controls. Significantly greater copy number of 16S rRNA and APase genes in root tips than mature zones rhizosphere microsites was revealed by qPCR analysis. Greater copy numbers and RA of *phoD* genes were also found in comparison with *phoX* genes. This study contribute to our understanding on how P fertilization may modulate the total and APase-harboring bacterial populations in the rhizosphere of plants. This knowledge might be relevant when P fertilizer practices are developed for pasture and crops established in Chilean Andisols. In order to improve the

plant P nutrition, the application of biofertilizers (e.g., plant growth-promoting rhizobacteria), engineering of the rhizosphere conditions, or selection of plant varieties could be defined according to their capability to promote the presence and activity of APase-harboring bacterial populations in the rhizosphere in a wide range of soils and environments.

4.6 Acknowledgments

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CHAPTER 5

General Discussion

5. General Discussion

The present thesis aimed at determining the composition of rhizobacterial communities in microsites of ryegrass (*Lolium perenne* var. Nui) grown in soil from grassland with low P availability under greenhouse conditions. In this context, our research was divided into three chapters, which considered an overview on the study of rhizobacterial ecology by modern molecular techniques, and an estimation of the changes on the diversity of rhizobacterial communities in microsites of ryegrass.

The rhizosphere, defined as soil zone affected by plant roots, is a complex environment where diverse microorganisms (bacteria, archaea, fungi, algae and protists) have an important role in nutrient cycling and plant growth (Bastidas et al., 2009). However, the structure and diversity of microbial communities along the rhizosphere differ in their composition and quantity according to diverse biotic and abiotic factors. In order to study bacterial communities along of rhizosphere, methods such as quantitative PCR, fingerprinting techniques (DGGE, TGGE, T-RFLP etc.), and high throughput sequencing (pyrosequencing) are being used to understand the role and functions of each bacterial group in the rhizosphere (van Elsas and Boersma, 2011; Nannipieri et al., 2008; Sørensen et al., 2009). Moreover, post–genomic techniques (metagenomics, metaproteomics and metatranscriptomics) are being applied to improve our understanding of the microbial communities at a higher resolution. In this context, in the Chapter 2 we presented an overview of the current knowledge on the study of bacterial community in the rhizosphere by using modern molecular techniques, describing classical molecular techniques, next generation sequencing platforms and post–genomics techniques. Concluding, there is still insufficient knowledge on microbial ecology in rhizosphere due to difficulties of extractions and purification of

nucleic acids and proteins from soil samples. Nevertheless, there are advances in sequencing techniques might allow discovery more genomes and functions of microbiota of bulk and rhizosphere soils.

Advances in nucleotide sequencing techniques might allow expanding libraries DNA or database to continue uncovering more genomes and functions of microbiota of soils. In this context, there is an exponential increase in the number of draft genomes reported in Genomes database. In fact, there are 30,437 genomes from three domains of life in the Joint Genome Institute´s Integrated Microbial Genomes database (Hug et al. 2016). By new bioinformatics methods is possible discovery new genomes which gives new information about phylogenetic and metabolic potential functions. As a consequence of these advances, Hug et al. (2016) presented a new view of the tree of life, highlighted a new microbial lineage, Candidate Phyla Radiation (CPR), which appears to subdivide the Bacteria domain and all members have relatively small genomes and restricted metabolic capacities (Fig. 5.1). We must consider not only at the level of bacteria there are new discovery at level taxonomy and function. Recently, Spribille et al. (2016) by transcriptome-wide analysis, discovered a new partner in the symbiotic association of lichens. Lichens are composed of the known ascomycete, the photosynthesizing partner, and, unexpectedly, specific basidiomycete yeasts, *Cyphobasidium* yeasts. The authors suggested that discovery of should change expectations about the diversity involved in one of the oldest known and most recognizable symbioses in soil.

Moreover, this research produced information on taxonomy composition of rhizobacterial at microsites level in grassland. Thus, throughout our research was possible evidence that each update database (Silva database) give new data for bacterial identification, improving information, minimizing data inconsistent. Data quality is a comprehensive approach to promoting the accuracy, validity, and timeliness of the data.

Table 5.1 shows the numbers of sequence identified which have been modified by Silva database update. Despite we do not detected differences in relation to taxa identification at genera level, the coverage, identity and similarity percentages were improved.

Table 5.1. Numbers of sequence identified with Silva database updated in April and September

	Silva Database	
	April,2015	September,2015
MZ_CT	17,973	15,380
MZ_PHO	18,857	16,707
MZ_PHY	24,558	20,843
RT_CT	18,989	15,883
RT_PHO	18,900	14,818
RT_PHY	22,513	18,552
Total of all groups is	**121,790**	**102,183**

On the other hand, one of the long term goals of rhizosphere biology research is to understand which factors are relevant (soil type, pH, nutrients availability, soil management) on microbial diversity along the root axes. Suzuki et al. (2009) indicated that bacterial communities are more dependent on soil type (Cumulic Andisol, Low-humic Andisol, Yellow Soil and Gray Lowland Soil) than on fertilizer type (chemical fertilizer, rice husk and manure). On the other hand, Fierer and Jackson, (2006) indicated that the diversity and richness of soil bacterial communities differ according to soil type and soil pH appears to be an important factor, demonstrating that bacterial diversity was higher in neutral soils and lower in acidic soils. In addition, Lauber et al. (2009) reveled that changes in the relative abundances of bacterial groups

(Acidobacteria, Actinobacteria, and Bacteroidetes) are also related to pH soil range. Other factor relevant has relation with the microbial diversity and abundance at the microsites influenced by nutrient availability, resulting competition among microbes. In addition, studies have purposed that addition P modulated the bacterial composition communities in the rhizosphere (Mander et al. 2012; DeForest and Scott, 2010).

Considering the factors which may influence on the ecology of the rhizosphere, in the Chapter 3 we assessed the structure of bacterial communities associated with selected microsites of the rhizosphere of ryegrass plants grown in naturalized pasture-Freire serie- and pasture under periodical fertilization -Piedras Negras series- under greenhouse conditions. Our results shown similar rhizobacterial communities for ryegrass plants grown in both Andisols. Moreover, high proportion of low-abundance of bacterial groups, including novel members do not described for soil and rhizosphere. These results contrasts with previous studies, which reported differences in bacterial community structures of rhizosphere microsites. These differences could be attributed to classical molecular techniques have lower resolution compared with 454 sequencing technique. Moreover, the absence of significant differences between microsites could due to differences in the strength of the rhizosphere effect among plant species (Lagos et al. 2014).

On the other hand, we know that Phosphorus (P) is an essential macronutrient, it is abundant in soils but their availability is limited for terrestrial organisms. In rhizosphere soil, under P deficiency, bacteria have shown capacity to solubilize Po and Pi, these processes are of special importance in the rhizosphere where the microbial activity can result in an increase of P availability, improving the P uptake with the consequent higher growth of plants, while plants reciprocate supplying C and energy to bacteria. Our knowledge about P fertilization effect on nutrients cycling, availability, and its

influence on ecology of rhizobacterial communities along roots still is poor. Therefore, in the Chapter 4 we assessed effect of P sources (phosphate and phytate) on rhizobacterial composition along roots of ryegrass and which its effect on abundance of functional genes encoding alkaline phosphomonoesterases (*phoX* and *phoD*). The same way to before experiment, our results shown not differences among rhizobacterial communities structure of ryegrass microsites, the bacterial dominant groups were Proteobacteria (members belonging to classes Alpha and Betaproteobacteria) followed by Actinobacteria, Cloroflexi and Acidobacteria. These dominant bacterial groups to shape a stable community despite to multiple factors as such abiotic and biotic which interfere among soils (Youssef and Elshahed 2009). Therefore, despite to kind of soil, agricultural management even, supplied P sources at soil, our results shown the same rhizobacterial community structure along roots. Nevertheless, our results revealed changes in abundance and richness of rhizobacterial community, according to DeForest et al. 2010, Fierer and Jackson, 2006, they attributed these changes of composition of the microbial community is directly rationed with available P, indicating that microbial communities in acidic soils are functionally P limited. Moreover, in relation the abundance of genes encoding alkaline phosphomonoesterase, ours results revealed a higher presence of *phoD* than *phoX* and a higher relative abundance of both genes in root tip in the phosphate treatment. Therefore, this last chapter revealed that P fertilization affects the microbial diversity of soil ecosystems, which might potentially modulate the P biogeochemical cycle and contribute to our understanding on how P fertilization may modulate the total and APase-harboring bacterial populations in the rhizosphere of plants.

Chapter 6

General Conclusions and Perspectives

6. General Conclusions and Perspectives

Metagenomics and metaproteomics approaches are allowing us to evaluate which organisms have which genes and which are expressed in the rhizosphere. To link these to the basic understanding of rhizobacterial composition at microsite level might allow us determine the controls on important soil processes such as nutrient cycling. In this context, the main objective of rhizosphere research is to elucidate the key biotic and abiotic factors that determine the composition and activity of the rhizobacterial. To reach understanding of the major drivers of rhizosphere processes can be exploited for the development of new strategies to promote plant growth or to control plant pathogens.

Our research shown a wide description of rhizobacterial community composition at microsites level presented in grassland established in Chilean Andisols, with this baseline information in these soils, we believe that is possible to develop of plant–microbial consortia that can enhance specific ecosystem processes such as nutrient bioavailability, promoting plant growth or disease suppression. Moreover, we have mentioned that strategies need to be developed that improve the P nutrient in our soils. Thus, our study contribute to our understanding on how P fertilization may modulate the bacterial populations in the rhizosphere of plants. This knowledge might be relevant when P fertilizer practices are developed for pasture and crops established in Chilean Andisols. Understanding and managing the rhizosphere might be an efficient strategy, and could be used to facilitate plant and microbial uptake of P and enhance crop access to P such as, the application of biofertilizers (e.g., plant growth-promoting rhizobacteria), engineering of the rhizosphere conditions, or selection of plant varieties could be defined according to their capability to promote the presence and activity of

APase-harboring bacterial populations in the rhizosphere in a wide range of soils and environments. We hope that application of our current knowledge about rhizosphere ecology provide tools for developing new agronomics practices for pasture and crops established in Chilean Andisols.

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Chapter 7

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7. REFERENCES

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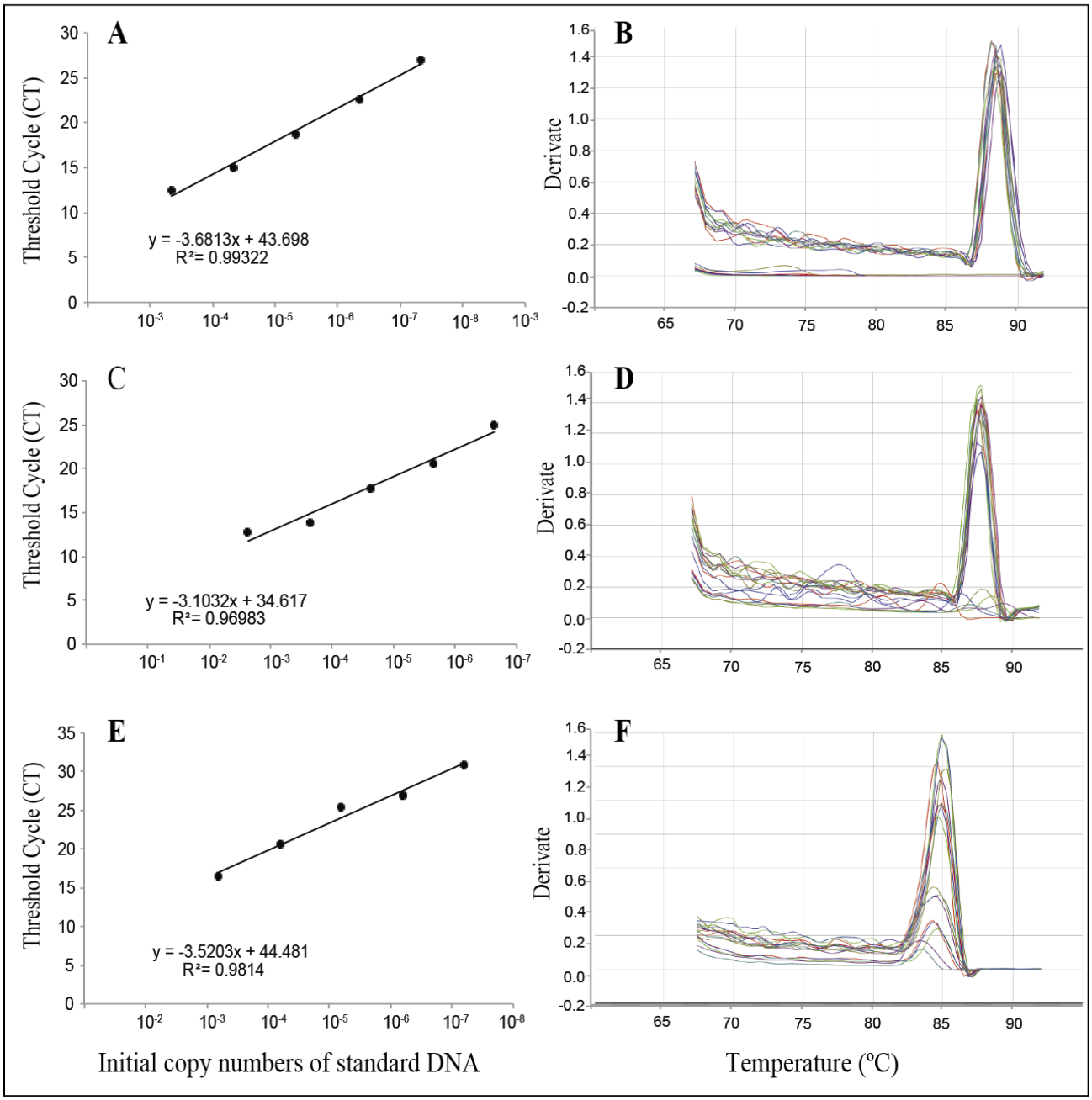
ANNEXES

Chapter 4. Effect of phosphorus addition on total and alkaline phosphomonoesterase-harboring bacterial populations in ryegrass rhizosphere microsites



Annex 8.1. Standard and dissociation curves of phoD (a and b), phoX (c and d) and 16S rRNA (e and f) genes used for qPCR analysis in the rhizosphere soil samples

Annex 8.2 Measurement pH in microsites samples.

8.2.1 Methodology

8.2.1.1 *Qualitative methodology*. According to experiment described in objective 2, one plant per triplicate from each treatment was selected. Roots with adhered soil, was pasted on surface of agarose (1%) gel film and then, bromocresol green (0.015%) pH indicator was dissolved in an small volume of ethanol and added to the gel. Yellow color indicates acid while purple color indicates alkalization (pH value range: <4 and>5.4).The initial pH was adjusted to 4.0 with a pH meter. After 1 h of incubation, color changes in the agarose gel was recorded by photography. For calculating pH values was used indexed color (RGB indexes) by Adobe Photoshop.

8.2.1.2 Quantitative methodology. According to experiment described in objective 2, microsites samples pH was measured in water (1:5 w:v, 0.2 g:1 mL). After of agitation by 10 m, the supernatant was measured for pH with a pH meter using a microelectrode, Thermo Orion. The data were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's minimum significant difference test.

8.2.2. Results

According to qualitative method for measurement pH using a pH indicator (green bromocresol), our results revealed difference between microsites samples in each treatment. In addition, our data shown a decrease on pH values in RT microsites of treatments P in comparison to RT microsite in CT treatment. In relation to MZ microsites of treatments P had an increase on pH values in comparison to MZ microsite in CT treatment (Fig. and Table 8.2.1). Moreover, we tested other methodology using a microelectrode for pH

measurement in microsites samples. According to quantitative methodology, our results showed difference values in comparison with before methodology. Nevertheless, our data revealed a similar tendency that before described. (Table 8.2.2).

Table 8.2.1 Measurement of pH microsites by pH indicator.

	CT		PHO		PHY	
Microsites	RT	MZ	RT	MZ	RT	MZ
pH value	4.33±0.17a	4.59±0.108a	4.45±0.15a	4.71 ±0.09a	4.58±0.08a	4.78±0.27a

Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil**.** Different letters indicate significant difference between samples. Determined by one-way ANOVA and post-hoc Tukey test ($P<0.05$, $n=3$).

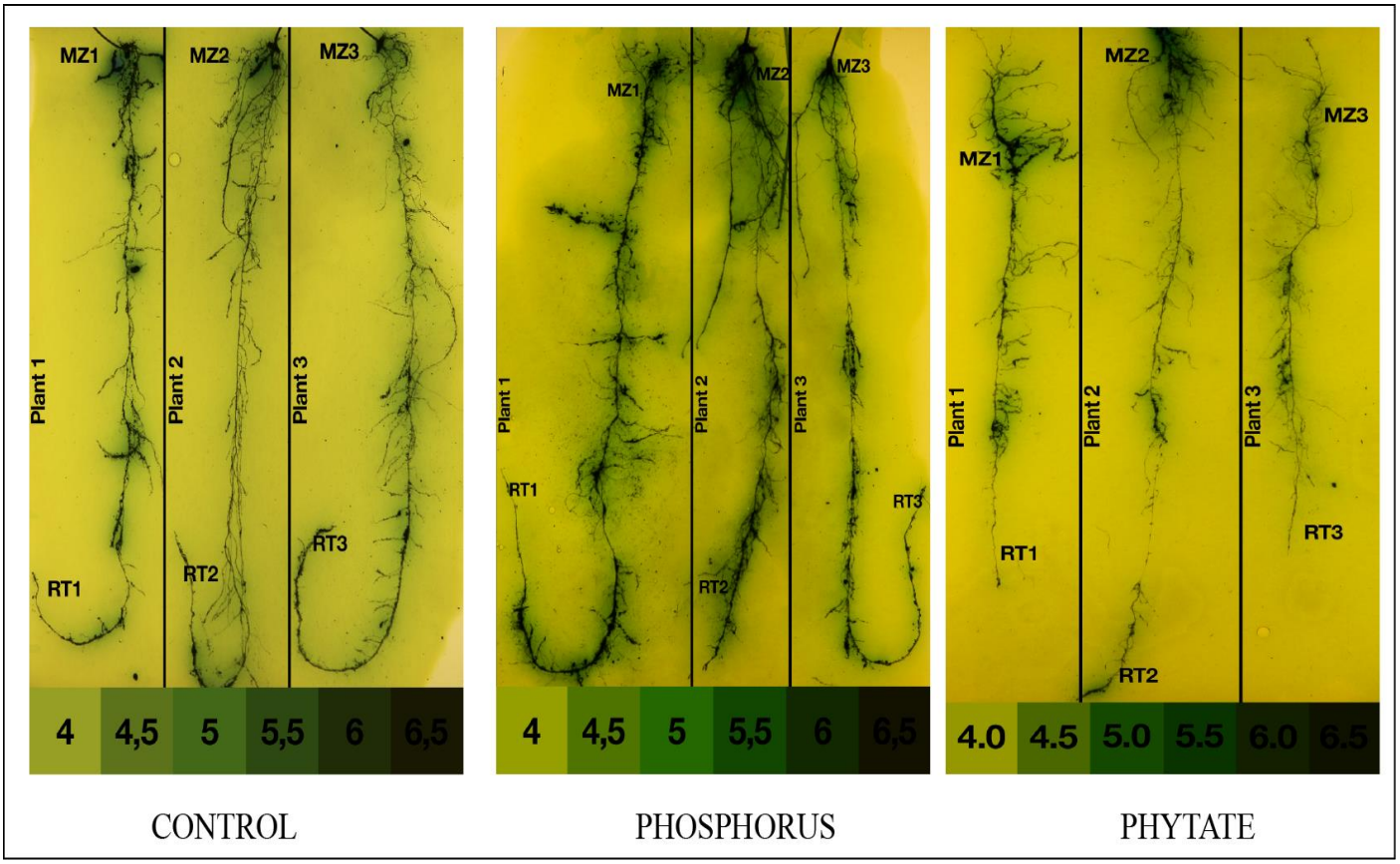


Figure 8.2.1 Changes of pH microsites of *Lolium perenne* var Nui after of 1 h of incubation in agarose gel film containing a pH indicator (green bromocresol 0.015%).

Table 8.2.2. Measurement of pH microsites by microelectrode.

	Control		Phosphorus source		Phytate Source	
Microsites	RT	MZ	RT	MZ	RT	MZ
pH value	6.32±0.09b	6.21±0.19b	5.57±0.18b	5.43±0.05b	5.61±0.17ab	5.38±0.06ab

pH rhizosphere was measured in water (1:5 w:v, 0.2 g:1 mL). Each abbreviation means RT, root tip; MZ, mature zone; CT, control treatment; PHO, phosphate treatment; PHY, phytate treatment; BULK, bulk soil**.** Different letters indicate significant difference between samples. Determined by one-way ANOVA and post-hoc Tukey test ($P<0.05$, $n=3$).