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INFLUENCE OF ENVIRONMENTAL FACTORS ON OCCURRENCE AND PGPR ACTIVITIES OF RHIZOBACTERIA IN VOLCANIC SOILS

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"INFLUENCE OF ENVIRONMENTAL FACTORS ON OCCURRENCE AND PGPR ACTIVITIES OF RHIZOBACTERIA IN VOLCANIC SOILS"

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I saw that wisdom is better than folly,
just as light is better than darkness.
The wise have eyes in their heads,
while fools walk in the darkness;
but I came to realize
that the same fate overtakes them both.
Then I said to myself,
“The fate of the fool will overtake me also.
What then do I gain by being wise?”
I said to myself,
“This too is meaningless.”
For the wise, like the fool, will not be long remembered;
the days have already come when both have been forgotten.
Like the fool, the wise too must die!

Eccles. 2:13-16

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Abstract

The plant growth promoting rhizobacteria (PGPR) have diverse mechanisms which play key roles in nutrient uptake, modulation of growth regulators and environmental stress tolerance in plants. Despite of many successful experiences, both in greenhouse and field, the application of PGPR on a commercial scale has frequently been problematic, mostly due to the low reproducibility of the results obtained for different crops. It has been suggested that the inconsistencies are the result of physical, chemical and biological soil properties, which could affect the establishment, survival and activity of the inoculated PGPR. There is little scientific information regarding environmental factors and agronomic management associated with volcanic acids soils that could affect PGPR performance as inoculants. The objectives of this study were to isolate and select native rhizobacteria capable of mobilizing insoluble forms of phosphorus (P) and synthesize indole acetic acid (IAA); in addition to determine the influence of typical environmental factors of Andisols on these mechanisms; and to evaluate the effect of rhizobacteria inoculation on plants in a low available P soil. In the first part of this project, we have isolated a total of 1,176 native bacterial strains from the rhizosphere of perennial ryegrass plants of a long-term trial plots with (N1: 600 kg N yr^{-1}) and without fertilization regime nitrogen (N0).The total isolated rhizobacteria were assessed its ability to solubilize phosphate, mineralize phytate and produce IAA in vitro. According to the results obtained, ten rhizobacteria strains were selected from each of the treatments (N0 and N1), which showed the highest in vitro efficiency for the three tested mechanisms. The selected rhizobacteria were identified by partial sequencing of 16S ribosomal RNA (rRNA). The selected rhizobacteria were members of the taxa *Enterobacter, Bacillus, Flavobacteria, Pseudomonas* and *Serratia*. Furthermore, the results revealed the N fertilization affected the occurrence of different types of potential culturable PGPR. Compared to the plot N0, N1 plot increased significantly (30%) the occurrence of culturable phosphobacteria, but decreased (7%) the incidence of IAA-producing rhizobacteria.

Regarding the influence of particular environmental factors on the activity of selected rhizobacteria, in vitro tests were performed to assess the effect of organic acids (citric, malic and oxalic acid at concentrations of 10, 50 and 100 μM), metals (Al and Mn at concentrations of 50, 200, 500 and 50, 100, 350 μM, respectively) and N sources (urea and ammonium sulfate at concentrations of 7.5, 15 and 30 mM) on the production indole acetic acid (IAA) and the release phosphorus (P). Four strains were selected (*Bacillus* sp. N1-19NA, *Enterobacter* sp. N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB) due to their greater capacity to produce IAA and to solubilize/mineralize P. At a low pH (4.8), the tests showed that IAA production by *Serratia* sp. N0-10LB increased (31 to 74%) in the presence of organic acids. Additionally, the production of IAA by *Pseudomonas* sp. N1-55PA increased by two to five times by the presence of metals. For all selected strains, growth and IAA production decreased significantly in the presence of 500 μM Al, with the exception of *Serratia* sp. N0-10LB, suggesting its potential as PGPR inoculants for acidic soils, such as Chilean Andisols. When urea was added as a main source of N, bacterial growth and the use of P significantly increased compared to when ammonium sulfate was added.

Subsequently, greenhouse trials were conducted using the four preselected strains mentioned above. Pot experiments were conducted to investigate the contribution of four selected rhizobacteria on the growth of cereals (wheat, oats and barley) grown in an Andisol of Piedras Negras Serie without P fertilization history. The following parameters were evaluated: plant biomass dry matter, P uptake and P concentration in plant tissue, rhizospheric soil enzyme activities, superoxide dismutase of root (SOD), and changes in bacterial communities in the rhizosphere. The result showed that *Enterobacter* N0-29PA significantly increased the P concentration (7.3%) in wheat plant (sterile soil), and plant dry biomass (29%) and P uptake (47%) of oat (non-sterile soil under P-deficient soil condition). In general, the inoculation with the others rhizobacteria (*Bacillus* sp. N1-19NA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB) did not incise the plant biomass, P uptake and P concentration in cereal plants compared with uninoculated control. Also, the inoculation treatment increased the available P in the rhizosphere (*Bacillus* sp. N1-19NA and *Enterobacter* sp. N0-

29PA) and resulted in changes in acid phosphatase and urease activity in the rhizosphere (*Pseudomonas* sp.N1-55PA in oats). In general, inoculation of all rhizobacteria increased (24-125%) the potential to produce IAA in the rhizosphere and inoculation of three of the four strains (*Enterobacter* sp. N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10 LB) increased the antioxidant activity (SOD) in wheat roots. In relation to the influence of inoculation on the structure of the native soil microbial community, DGGE fingerprint analysis revealed no consistent differences between treatments within cereal species.

The present study shows that environmental factors and agricultural management (pH, metals, fertilizer N) can influence the occurrence and activity of rhizobacteria associated to plant growth promoting mechanisms.The inoculation of rhizobacteria in volcanic soil P-deficient, can influence important plant growth parameters and abiotic stress tolerance, such as rhizosphere soil available P, the enzymatic activities of the rhizosphere (acid phosphatase and urease) and plants antioxidant activity (SOD).

Resumen

Las rizobacterias promotoras del crecimiento vegetal (conocidas por su sigla en inglés *PGPR*, plant growth promoting rhizobacteria) tienen diversos mecanismos que desempeñan roles claves en la absorción de nutrientes, modulación de reguladores de crecimiento y tolerancia al estrés ambiental en plantas. A pesar de muchas experiencias exitosas, tanto en invernaderos y campo, la aplicación de PGPR a escala comercial no ha sido fácil, debido a la baja reproducibilidad de los resultados obtenidos en diferentes cultivos. Se ha sugerido que las inconsistencias son consecuencia de las características físicas, químicas y biológicas del suelo, lo que podría afectar el establecimiento, supervivencia y actividad de las PGPR inoculadas. En el caso de nuestro país, existe poca información con respecto a los factores ambientales y de manejo agronómico asociados a suelos volcánicos ácidos que podrían afectar el desempeño de PGPR como inoculantes. Los objetivos de este estudio fueron aislar y seleccionar rizobacterias nativas con capacidad de movilizar formas insolubles de fósforo (P) y sintetizar ácido indol acético (AIA); además de determinar la influencia de factores ambientales propios de suelos Andisols sobre estos mecanismos; y evaluar la inoculación de rizobacterias en plantas en un suelo deficiente en P disponible. En la primera parte de este proyecto, se aisló un total de mil ciento setenta y seis cepas bacterianas nativas fueron aisladas desde la rizósfera de parcelas con *Lolium perenne,* con (N1: 600 kg de N año^{-1}) y sin régimen de fertilización con nitrógeno (N0) en ensayo de largo plazo. Al total de rizobacterias aisladas se les evaluó su capacidad para solubilizar fosfato, mineralizar fitato y producir AIA in vitro. De acuerdo a estos ensayos, se seleccionaron diez rizobacterias provenientes de cada uno de los tratamientos (N0 y N1) y que presentaron la mayor eficiencia en los tres mecanismos evaluados. Las rizobacterias seleccionadas fueron caracterizadas mediante la secuenciación parcial del gen 16S ARN ribosomal (rRNA). Las rizobacterias fueron caracterizadas como miembros de las taxa *Enterobacter*, *Bacillus*, *Flavobacteria*, *Pseudomonas* y *Serratia*. Además, los resultados revelaron la influencia de la fertilización con N sobre la ocurrencia de potenciales PGPR cultivables. En comparación con la parcela N0, la parcela N1 incrementó significativamente (30%)

la aparición de fosfobacterias cultivables, pero disminuyó (7%) la incidencia de rizobacterias productoras de AIA.

En relación la influencia de factores ambientales particulares de suelos Andisols sobre la actividad de las rizobacterias seleccionadas, se evaluó in vitro los efectos de los ácidos orgánicos (cítrico, málico y oxálico en concentraciones de 10, 50 y 100 μM), metales (Al y Mn en concentraciones de 50, 200, 500 y 50, 100, 350 μM, respectivamente) y de fuentes de N (urea y sulfato de amonio en concentraciones de 7,5, 15 y 30 mM) sobre la producción ácido indol acético (AIA) y la liberación fósforo (P) de cuatro cepas seleccionadas. Las cepas fueron seleccionadas (*Bacillus* N1-19NA, *Enterobacter* N0-29PA, *Pseudomonas* N1-55PA y *Serratia* N0-10LB) por su mayor capacidad para producir AIA y solubilizar/mineralizar P. A un pH bajo (4,8), los ensayos mostraron que la producción de AIA por *Serratia* sp. N0-10LB se incrementó (31 a 74%) en presencia de los ácidos orgánicos. Por otro lado, la producción de AIA por *Pseudomonas* sp. N1-55PA se incrementó en dos a cinco veces por la presencia de metales. En todas las cepas, el crecimiento y la producción de AIA disminuyeron significativamente en una concentración de 500 μM Al, con excepción de *Serratia* sp. N0-10LB, lo que sugiere su potencial como PGPR para ser utilizada en suelos ácidos, como los Andisoles chilenos. Cuando la urea se agregó como principal fuente de N, el crecimiento bacteriano y la utilización de P aumento significativamente en comparación a cuando se agregó sulfato de amonio.

Posteriormente, se realizaron ensayos en invernadero donde se utilizaron las cuatro cepas mencionadas arriba. Experimentos en macetas se llevaron a cabo para investigar la contribución de las cuatro rizobacterias seleccionadas sobre el crecimiento de cereales (trigo, avena y cebada) cultivados en un Andisol de la serie Piedras Negras sin historial de fertilización con P. Se evaluaron los siguientes parámetros biomasa vegetal en base materia seca, absorción de P y la concentración de P en el tejido de las plantas, actividades enzimáticas del suelo rizosférico, superóxido dismutasa de la raíz (SOD) y las comunidades bacterianas de la rizósfera. Los resultados mostraron que *Enterobacter* N0-29PA aumentó significativamente la concentración de P (7,3%) en trigo (suelo estéril), y la biomasa seca de la planta (29%) y la absorción de P (47%) en

avena (suelo no estéril, sin fertilización con P). En general, la inoculación con las otras rizobacterias (*Bacillus* sp. N1-19NA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB) no afecto la biomasa vegetal, ni la concentración de P en plantas de cereales en comparación con el control sin inocular. Además, la inoculación de las cepas aumentó el P disponible en la rizósfera (*Bacillus* N1-19NA y *Enterobacter* N0-29PA) y produjo cambios en las actividades enzimáticas fosfatasa ácida y ureasa en la rizósfera (*Pseudomonas* sp.N1 55PA en avena). En general, la inoculación de todas las rizobacterias incrementó (24 a 125%) el potencial de producir AIA en la rizósfera y la inoculación de tres de las cuatro cepas (*Enterobacter* sp. N0-29PA, *Pseudomonas* sp.N1 55PA y *Serratia* sp. N0-10 LB) aumentó la actividad antioxidante de la enzima SOD enla raíz trigo. En relación a la influencia de la inoculación sobre la estructura de la comunidad bacteriana nativas del suelo, los resultados de DGGE no revelaron diferencias consistentes entre los tratamientos en cada uno de las plantas de cereal.

El presente estudio muestra que factores ambientales y de manejo agronómico (pH, metales, fertilización N) puede condicionar la ocurrencia y actividad de rizobacteria con mecanismos asociados a la promoción del crecimiento vegetal y que la inoculación de rizobacterias en suelo volcánico con deficiencia de P, puede influenciar parámetros relevantes para el crecimiento vegetal y tolerancia al estrés abiótico, tales como P disponible del suelo rizosferico, las actividades enzimáticas de la rizósfera (fosfatasa ácida y ureasa) y plantas (SOD).

List Abbreviations

°C	Degrees celsius
µ	Micro
ACC	1-aminocyclopropane-1-carboxylate
Al^{+3}	Aluminum ion
ANOVA	Analysis of variance between groups
AS	Ammonium sulphate
BLAST	Basic local alignment search tool
CFU	Colony forming units
CP	Tricalcic phosphate solubilization
DAPG	2,4-diacetyl phloroglucinol
DGGE	Denaturing gradient gel electrophoresis
Fe	Iron
IAA	Indole acetic acid
IAAP	Indole acetic acid-producing phosphobacteria
ha	Hectare
HCN	Hydrogen cyanide
kg	Kilogram
l	Liter
LB	Luria-Bertani agar
L-TRP	L-tryptophan
ml	Milliliter
M	Molar
Mn^{+2}	Manganese ion
N	Nitrogen
NA	Nutrient agar
NBRIP	National Botanical Research Institute's phosphate growth medium
NH_4^+	Ammonium ion
NBT	Nitroblue tetrazolium
OD	Optical density
P	Phosphorus

PAB	*Pseudomonas* agar base
PGPR	Plant growth promoting rhizobacteira
PM	Phosphate mineralization
p-NPP	*p*-nitrophenylphosphate
p-NP	*p*-nitrophenyl
PS	Phosphate solubilization
PSM	Phytase-screening medium
PCR	Polymerase chain reaction
PGPR	Plant Growth Promoting Rhizobacteria
R2A	R_2A agar
rRNA	Ribosomal ribonucleic acid
SID	Siderophore production
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive-substances
U	Urea
V	Volt
yr	Year

Chapter 1

General Introduction

General Introduction

Plant growth promoting rhizobacteria (PGPR) are defined as free living bacteria that colonize and live in the roots that exert beneficial effects on growth, disease suppresion and stress tolerance of plants. Among the principal mechanisms by which PGPR promote plant growth are the capacity to increase phosphorus (P) and nitrogen (N) availability, production of indole acetic acid (IAA), production of antibiotics and induction of systemic resistance in plants. Under this scenario, PGPR are being considered as a significant component to achieve sustainable crop yields by reducing the use of pesticides and making more efficient the use of chemical fertilizers.

Experiments in, both greenhouses and fields have demonstrated that the application of PGPR in major scale has not been easy, due to the low reproducibility of the results obtained at the field test level in the different crops. This limitation can be a consequence of physical, chemical and biological soil characteristics, such as the soil texture, pH, nutrient status, humidity, temperature, organic matter content and biological interactions of the rhizosphere, which could affect the establishment, survival and activity of the inoculated PGPR (Lambert and Joos 1989; Richardson 2001; Rengel 2008). This aspect has limited the commercial development inoculants and makes necessary to better understand the influence that environmental factors on the beneficial mechanisms of rhizobacteria on plants which inoculant is applied.

In Chile, PGPR represents an interesting alternative for agriculture, which is characterized by a high dependency in agrochemicals (synthetic chemical fertilizers and biocides). Chemical fertilizers increase the production costs and cause constant contamination on the environment. Nowadays, PGPR as inoculant are not used in traditional agriculture, such as pasture and cereal crops in southern of Chile, but there is a need for a more efficient use of fertilizer by crops, particularly phosphorus (P). In this

context, PGPR known as phosphobacteria arise an alternative to P-fertilizers. Phosphobacteria is refered to bacteria that living in the rhizosphere have the ability to convert insoluble P (both inorganic and organic) to an accessible form to plants. The predominant soils in southern Chile are those derived from volcanic ash (Andisols) and a large percentage of the agricultural productions are developed in these soils. Andisols are characterized by acid soils (pH < 5.5) and high content of total P, product of the prolonged applications of P-fertilizers, however, their availability for the plants is very low. This is mainly caused because some of the soluble P applied to soils as fertilizer is rapidly absorbed by the colloidal fractions of the soil and becomes unavailable to plants (Rodriguez and Fraga 1999). Organic P is one of major P forms in soils comprising between 30 and 50% of the total P in many soils, being phytate as predominant form (30-60% of organic P) (Richardson 2001; Turner 2007). On the other hand, the Andisols are characterized by a high metal phytotoxic cations (Al^{+3} and Mn^{+2}) in the soil solution (Inostroza-Blancheteau et al. 2008; Millaleo et al. 2010) and low pH, mainly caused by precipitations and N fertilization applications such as urea (Mora et al. 2002).

The potential use of PGPR in Chilean Andisols requires a better understanding of the influence of endemic environmental factors on their effectiveness, such as pH, cation presence (Al and Mn), low levels of available P and crop management, could have over the potential performance of inoculate rhizobacteria.

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Hypotheses

Hypothesis I:

In the rhizosphere of pasture systems developed in volcanic acid soils of southern of Chile, exist native rhizobacteria carrying PGPR activities which are modulated by environmental factors such as nitrogen, organic acid, cation metals present in acidic soil solution.

Hypothesis II:

Based on the ability to utilize insoluble forms of phosphorus and to produce indole acetic acid, rhizobacteria can stimulate growth of cereal plants under P-deficient soil conditions.

General objective

To study the influence of environmental factors, such as nitrogen, organic acids, cation metals present in soil solution, on occurrence and PGPR activities of rhizobacteria in a volcanic soils.

Specific objectives

1. To isolate native rhizobacteria from pasture systems of acid volcanic soils of southern Chile.
2. To select phenotypically and genotypically native rhizobacteria presenting multiple mechanisms associated with the promotion of plant growth, such as the use of insoluble forms of phosphorus (P solubilizing and P mineralizing) and production of indole acetic acid.
3. To to evaluate in vitro the effects of organic acids (citric, malic and oxalic acids), metals (aluminium and manganese), and nitrogen supply (ammonium sulphate and urea) on the potential PGPR activities (indole acetic acid [IAA] production and P liberation) of selected IAA-producing phosphobacteria.
4. To evaluate the contribution of selected rhizobacteria to stimulate plant growth of cereals under P-deficient soil condition in greenhouse.

Chapter 2

Review

Mechanisms and Practical Considerations Involved in Plant Growth Promotion by Rhizobacteria

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Abstract

Rhizobacteria are capable of stimulating plant growth through a variety of mechanisms that include improvement of plant nutrition, production and regulation of phytohormones, and suppression of disease causing organisms. While considerable research has demonstrated their potential utility, the successful application of plant growth promoting rhizobacteria (PGPR) in the field has been limited by a lack of knowledge of ecological factors that determine their survival and activity in the plant rhizosphere. To be effective, PGPR must maintain a critical population density of active cells. Inoculation with PGPR strains can temporarily enhance the population size, but inoculants often have poor survival and compete with indigenous bacteria for available growth substrates. PGPR often present more than one mechanism for enhancing plant growth and experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms of action that may be activated simultaneously. The aim of this review is to describe PGPR modes of action and discuss practical considerations for PGPR use in agriculture.

Keywords: Agricultural inoculant, phytohormone, phytopathogen biocontrol, plant nutrition, rhizosphere.

2.1. **Introduction**

Plant growth promoting rhizobacteria (PGPR) influence plant health and productivity by a variety of mechanisms that involve solubilization of mineral nutrients, stimulation of root growth, and suppression of root diseases. Since the first studies on PGPR in the 1950's, many hundreds of candidate PGPR strains have been screened and evaluated in laboratory, greenhouse and field studies across the world. Today PGPR are commonly used in developing countries, and inoculants are used on millions of hectares of land (Zehnder et al. 2001). Nevertheless, implementation of this biotechnology has been hindered by the lack of consistency and variation in responses that are obtained in field trials from site to site, year to year, or for different crops (Lambert and Joos 1989). Successful establishment of the introduced bacteria depends on proper PGPR selection that must be tailored to the soil and crop combination. Other basic problems that are related to inoculum production, storage, and delivery have mostly precluded the use of non-spore forming bacteria as soil inoculants. Lastly, there has been considerable confusion over the precise PGPR effects which confound scientific studies aimed at quantifying their contribution to plant growth. This is largely due to poor understanding of the interactions between PGPR and their plant hosts and the resident microflora, as well as a paucity of information on how environmental factors influence processes that contribute to plant growth promotion.

Two paradigms that have emerged so far from the study of PGPR is that many of the best strains are multifunctional, and secondly, that PGPR traits are commonly distributed among many different species and genera of microorganisms, many of which are indigenous members of the soil microbial community. In most cases, individual strains vary considerably in performance and there is no clear relationship

between taxonomy and PGPR functions that can be used to monitor the population size and activity of these bacteria based on quantification of specific taxonomic groups in the soil. The possibility that indigenous PGPR affect the relative performance of introduced PGPR inoculants is quite high, so without knowledge of background PGPR activity, the response to soil inoculation is difficult to predict. Many PGPR simultaneously solubilize phosphorus, produce auxins that stimulate root growth, and produce antibiotics and siderophores that may function in suppression of root disease. Other traits that may contribute to plant growth promotion include production of substances that induce systemic resistance, or enzymes degrading hydrogen cyanide or ethylene and reactive oxygen species that are produced by plants during environmental stress. Lastly, the phenomenon of quorum regulation can affect the expression of each of these traits as PGPR interact with the resident microbial community (reviewed by Lugtenberg and Kamilova 2009). In this manner, critical threshold population sizes are likely required to induce the expression of some traits, particularly those involved in biocontrol. Altogether any and all of the cumulative effects of PGPR that influence root growth rates, root system architecture, root hair formation and longevity, will indirectly affect the ability to acquire water and nutrients and to tolerate root loss to disease. Deciphering which mechanisms are most important and how to manage the soil microflora to obtain expression of these traits is so the remaining great challenge for consistent PGPR use in agricultural systems.

In this review, we examine the types of PGPR bacteria that have been identified to date and their functional characteristics. We also examine briefly inoculum production and delivery technologies and the advantages and disadvantages of various methods for introducing and maintaining high population densities of PGPR that are needed in order to be effective.

2.2. **General characteristics of PGPR**

PGPR have been subjected to numerous investigations focused on biotechnological applications in agriculture, horticulture, forestry and environmental protection (Zahir et al. 2004). Early studies in the 1950's began with a focus on nitrogen fixing bacteria. Since then, a large number of PGPR belonging to different bacterial classes and genera with multifunctional traits have been described (Rodríguez-Díaz et al. 2008). PGPR strains are broadly distributed among many taxa including *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* (Tilak et al. 2005), such that determination of the background population size and activity of PGPR in resident microbial communities is difficult to assess based on analysis of microbial community structure or abundance of a particular taxonomic group. The main aim of biotechnological development based on PGPR has been to develop soil inoculants that can contribute to a sustainable agriculture, thereby diminishing the need for use of chemical fertilizers and pesticides (Adesemoye and Kloepper 2009).

Based on our present knowledge, the interactions between bacteria and plants can be classified into three categories: neutral, negative or positive (Whipps 2001). Most rhizobacteria associated with plants are commensals, in which bacteria establish an innocuous interaction that does not have any visible effect on the growth and physiology of the plant (Beattie 2006). The rhizosphere also contains rhizobacteria that negatively influence the growth and physiology of the plants, and includes phytopathogens (Beattie 2006). In addition to parasitic and disease causing organisms, such bacteria include those that produce phytotoxic substances, such as hydrogen cyanide or ethylene that inhibit root growth. Counter to these deleterious bacteria are PGPR, which exert a positive effect on plant growth by direct mechanisms such as

solubilization of nutrients, nitrogen fixation, production of growth regulators, etc., or by indirect mechanisms such as stimulation of mycorrhizae development, competitive exclusion of pathogens, or removal of phytotoxic substances that are produced by deleterious bacteria and plant roots under stress condition mechanisms (Beattie 2006; Bashan and de-Bashan 2010).

In addition to these functional classifications, PGPR can be further grouped with respect to the plant compartment that they occupy as either intracellular (iPGPR, symbiotics) or extracellular (ePGPR, free living), in accordance with the degree of association with the root cells. The iPGPR may live inside the root cells, generally in specialized structures, such as nodules. Extracellular ePGPR are situated either in the rhizosphere, on the root surface (rhizoplane) or in the intercellular spaces of the root cortex, colonizing the plant tissue intercellularly (Gray and Smith 2005).

In accordance with the mechanisms presented by PGPR, classification terms have been established (Table 2.1) to describe their activities and mechanisms by which these functions are achieved. In general, direct mechanisms are those affecting the balance of plant's growth regulators, enhancing plant's nutritional status and stimulating systemic disease resistance mechanisms (Zahir et al. 2004; Glick et al. 2007). Indirect mechanisms are related to biocontrol, including antibiotic production, chelation of available Fe in the rhizosphere, synthesis of extracellular enzymes that hydrolyze the fungal cellular wall and competition for niches within the rhizosphere (Zahir et al. 2004; Glick et al. 2007). This classification has led to the application of generic terms including: biofertilizer, phytostimulator and biopesticide to describe the primary function. Nonetheless, many bacteria have dual roles, which can lead to confusion. The best example of such confusion is found in the body of work on *Azospirillum*, which initially was based on this bacterium's ability to fix nitrogen, but which was later shown

to affect plant growth by production of phytohormones. Since then, it has been classified primarily as a phytostimulator (Okon and Kapulnik 1986; Spaepen et al. 2008). Similarly, many phosphorus-solubilizing bacteria have been screened and selected based on their ability to solubilize hydroxyapatite on agar media, but they have later been found to affect root growth by production of plant growth hormones. Despite the confusion generated by multifunctional PGPR, it is worthwhile to examine the traits associated with each of the three generic descriptors that are used to classify PGPR.

2.3. **Contribution of rhizobacteria to plant nutrition**

Microorganisms having mechanisms that facilitate nutrient uptake or increase nutrient availability or stimulate plant growth are commonly referred to as biofertilizers. Biofertilizers are considered as an alternative or complement to chemical fertilization to increase the production of crops in low input agricultural systems. There are some PGPR that can fix nitrogen, solubilize mineral nutrients and mineralize organic compounds. The most well-studied PGPR considered biofertilizers correspond to nitrogen fixation and utilization of insoluble forms of phosphorus.

2.3.1. Agronomic significance of biological nitrogen fixation

Nitrogen (N) is one of the principal plant nutrients, and its low availability due to the high losses by emission or leaching is a limiting factor in agricultural ecosystems, hence bacteria with ability to make atmospheric N available for plants play a critical role. There are two types of biological fixation: symbiotic and non-symbiotic. The first is the most important mechanism by which most atmospheric N is fixed, but it is limited to

legume plant species and various trees and shrubs that form actinorrhizal roots with *Frankia*. This process is carried out in well defined nodule structures. Among the most studied symbiotic bacteria are *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Mesorhizobium* (Zahran 2001). Although the beneficial effects of the symbiotic association of rhizobia with legume plants is known, these bacteria are not considered PGPR, except when associated with non-legume plants (Dobbelaere et al. 2003). On the other hand, non-symbiotic biological N fixation, is carried out by free living diazotrophics, and this can stimulate non-legume plants growth (Antoun et al. 1998). There are studies showing that N-fixing bacteria, free-living as well as *Rhizobium* strains, can stimulate the growth of non-legumes such as radish (Antoun et al. 1998) and rice (Mirza et al. 2006), in this way contributing to reduced dependence on N-based fertilizers (Bhattacharjee et al. 2008). Non-symbiotic N-fixing rhizospheric bacteria belonging to genera including *Azoarcus* (Reinhold-Hurek et al. 1993), *Azospirillum* (Bashan and de-Bashan 2010), *Burkholderia* (Estrada de los Santos et al. 2001), *Gluconacetobacter* (Fuentes-Ramírez et al. 2001) and *Pseudomonas* (Mirza et al. 2006) have been isolated from different soils.

Due to the high energy requirement for N fixation and relatively low metabolic activity of free living organisms that must compete for root exudates outside a nodule environment, the ability of nonsymbiotic bacteria to fix significant quantities of nitrogen is limited. The presence of a diazotrophic bacterium in the rhizosphere of a certain plant is no longer considered to imply that such bacteria make a substantial contribution to N fixation and N supply for plant growth. Although the N fixing capacity of certain bacteria can easily be demonstrated under *in vitro* conditions, its demonstration in greenhouse and field studies is more complex and highly variable. Some observations suggest that rhizobacteria can provide crops with significant

quantities of N (Dobbelaere et al. 2003). Nevertheless, studies in sorghum, maize and wheat inoculated with *Azospirillum* have revealed a contribution of only 5 kg N ha^{-1} yr^{-1} (Okon and Lanbandera-Gonzalez 1994). This quantity pales in importance when compared with the application of N fertilizers in a range of 150-200 kg N ha^{-1} yr^{-1}, which is commonly practiced in modern agriculture. This applies likely to other free living N fixers. Recently, Unkovich and Baldock (2008) pointed out that the contribution of N by free living soil bacteria for crop growth in Australia is probably <10 kg ha^{-1} yr^{-1}. Peoples et al. (2002) present a N fixation value of 0 to 15 kg ha^{-1} yr^{-1} and Bottomley and Myrold (2007) suggest annual values between <1 and 10 kg ha^{-1}. For this reason, the ability of PGPR to fix N is no longer an important criterion for classification of a bacterium as a biofertilizer.

Table 2.1. Terms adopted for classified mechanisms by which plant growth promoting bacteria stimulate plant growth.

Term	Definition	Mechanisms	References
Biofertilizer	A substance which contains live microorganisms which, when applied on the seed, plant surface or the soil, colonizes the rhizosphere or the interior of the plant and promotes growth through increased supply or availability of primary nutrients for the host plant.	- Biological nitrogen fixation - Utilization of insoluble forms of phosphorus	Vessey 2003; Somers et al. 2004; Fuentes-Ramírez and Caballero-Mellado 2006
Phytostimulator	Microorganism with the ability to produce or change the concentration of growth regulators such as indole acetic acid, gibberellic acid, cytokinins and ethylene.	- Production of phytohormones (auxins, cytokinins and gibberelins) - Decreased ethylene concentration (in the interior of the plant)	Lugtenberg et al. 2002; Somers et al. 2004
Biopesticide or biocontrol agent	Microorganisms that promote plant growth through the control of phytopathogenic agents, mainly for the production of antibiotics and antifungal metabolites.	- Production of antibiotics (siderophores, HCN, antifungal metabolites) - Production of enzymes that degrade the cellular wall of the fungi - Competition for sites in the root - Acquired and Induced systemic resistance	Vessey 2003; Somers et al. 2004; Chandler et al. 2008

2.3.2. Enhancing phosphorus availability for plant growth by rhizobacteria

Phosphorus (P) is an essential plant nutrient with low availability in many agricultural soils. Today many agricultural soils have a high total P content due to the application of P fertilizers over long periods of time. On the other hand, much of this P is in mineral forms and is only slowly available to plants (reviewed by Rodríguez et al. 2006 and Richardson et al. 2009). Most of the insoluble P forms are present as aluminum and iron phosphates in acid soils (Mullen 2005), and calcium phosphates in alkaline soils (Goldstein and Krishnaraj 2007). The ability of rhizosphere bacteria to solubilize insoluble P minerals has been attributed to their capacity to reduce pH by the excretion of organic acids (e.g. gluconate, citrate, lactate and succionate) and protons (during the assimilation of NH_4^+) (Gyaneshwar et al. 1999; Mullen 2005). These bacteria have been characterized as members of the *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Kluyvera*, *Streptomyces*, *Pantoea* and *Pseudomonas* genera (Chung et al. 2005; Hariprasad and Niranjana 2009; Oliveira et al. 2009) in various studies of P solubilizing bacteria from different rhizospheric soils. These microorganisms grow in media with tricalcium phosphate or similar insoluble materials as the only phosphate source and not only assimilate the element, but also solubilize quantities in excess of their nutritional demands, thereby making it available for plants (Chen et al. 2006).

On the other hand, organic P can constitute between 30 and 50% of the total P of the soil, a high proportion of it corresponding to phytate (Borie et al. 1989; Turner et al. 2002). In this context, there are bacteria capable of producing phytase enzymes for the mineralization of phytates (Lim et al. 2007; Jorquera et al. 2008b). To date, there are only few studies reporting rhizobacteria capable of mineralizing the phytate. Among the phytase producing rhizobacteria, species belonging to *Bacillus*, *Burkholderia*,

Enterobacter, *Pseudomonas*, *Serratia* and *Staphylococcus* genera are the most common culturable bacteria (Richardson and Hadobas 1997; Hussin et al. 2007; Shedova et al. 2008). Many of these bacteria are remarkably efficient. Richardson and Hadobas (1997) isolated *Pseudomonas* spp. that utilized phytate from different soils in Australia. The isolated strains exhibited a high phytase activity, releasing over 80% of the P content in the phytate. In a later study utilizing plants with a limited capacity to obtain the P from phytate, Richardson et al. (2001) observed that the ability of pasture plants to acquire P from phytate was enhanced followed by inoculation with the specified *Pseudomonas* sp. strains. Similarly, Unno et al. (2005), isolated diverse bacteria with the ability to utilize phytate from the rhizosphere from white lupin (*Lupinus albus*). Almost all the isolates were classified as members of the *Burkholderia* genus and some of them significantly promoted the growth of the lupin. Jorquera et al. (2008a) isolated P solubilizing bacteria from the rhizospheres of five cultivated plants (*Lolium perenne*, *Trifolium repens*, *Triticum aestivum*, *Avena sativa*, *Lupinus luteus*), which presented more than one mechanism for utilizing insoluble forms of phosphorus. Moreover, all strains showed the capacity to produce P hydrolases. The major limitation today for use of these organisms is the lack of consistent effects in mobilizing P under field conditions. This is likely due to competition with the native microflora and environmental factors that either limit the population size or activity of the PGPR. It is now clear from many studies that evaluation and ranking of P-solubilizing bacteria under laboratory conditions do not necessarily correspond to the efficacy of the PGPR for enhancing plant P uptake under field conditions (Richardson 2001; Rengel 2008). As with nitrogen fixing bacteria, the production of plant growth hormones that improve root surface area can have indirect effects on the ability to efficiently extract P from soil. Thus, it is likely that many so-called biofertilizers have dual action effects that are mediated by direct

solubilization of inorganic P, mineralization of organic P, and stimulatory effects on plant root growth or mycorrhizae formation.

2.4. **Production of phytohormones and regulation of ethylene levels in plant**

The production of phytohormones by PGPR is now considered to be one of the most important mechanisms by which many rhizobacteria promote plant growth (Spaepen et al. 2007). Phytohormones are signal molecules acting as chemical messengers and play a fundamental role as growth and development regulators in the plants. Phytohormones are organic compounds that in extremely low concentrations influence biochemical, physiological and morphological processes in plants, and their synthesis is finely regulated (Fuentes-Ramírez and Caballero-Mellado 2006). Numerous fungal and bacterial species can produce phytohormones (Tsavkelova et al. 2006). The phytohormone producing ability is widely distributed among bacteria associated with soil and plants. Studies have demonstrated that the PGPR can stimulate plant growth through the production of auxins (indole acetic acid) (Spaepen et al. 2008), gibberellines (Bottini et al. 2004) and cytokinins (Timmusk et al. 1999), or by regulating the high levels of endogenous ethylene in the plant (Glick et al. 1998).

2.4.1. Indole acetic acid (IAA) producing rhizobacteria

Some of the most abundant phytohormones present in nature are the auxins, IAA being the main plant auxin. IAA is responsible for the division, expansion and differentiation of plant cells and tissues and stimulates root elongation. The ability to synthesize IAA

has been detected in many rhizobacteria as well as in pathogenic, symbiotic and free living bacterial species (Costacurta and Vanderleyden 1995; Tsavkelova et al. 2006).

At present, auxin synthesizing rhizobacteria are the most well-studied phytohormone producers (Tsavkelova et al. 2006; Spaepen et al. 2007). These rhizobacteria synthesize IAA from tryptophan by different pathways, although it can also be synthesized via tryptophan-independent pathways, though in lower quantities (Spaepen et al. 2007). Phytopathogenic bacteria mainly use the indole acetamide pathway to synthesize IAA, which has been implicated in tumor induction in plants. It is not clear whether it is used by beneficial bacteria. In contrast, the acid indole pyruvic pathway appears to be the main pathway present in plant growth promoting beneficial bacteria (Patten and Glick 2002).

Among PGPR species, *Azospirillum* is the best-known IAA producer (Dobbelaere et al. 1999). Others IAA producing bacteria belonging to *Aeromonas* (Halda-Alija, 2003), *Azotobacter* (Ahmad et al. 2008), *Bacillus* (Swain et al. 2007) *Burkholderia* (Halda-Alija 2003), *Enterobacter* (Shoebitz et al. 2009), *Pseudomonas*(Hariprasad and Niranjana 2009) and *Rhizobium* (Ghosh et al. 2008) genera have been isolated from different rhizosphere soils.. Inoculation with IAA producing PGPR has been used to stimulate seed germination, to accelerate root growth and modify the architecture of the root system, and to increase the root biomass. In recent studies, Tsavkelova et al. (2007) have extended beyond individual strains as inoculants and reported an increase in the germination of orchid seeds (*Dendrobium moschatum*) inoculated with *Sphingomonas* sp. and IAA producing *Mycobacterium* sp. In addition to stimulating root growth, IAA producing bacteria can also be used to stimulate tuber growth. Swain et al. (2007) reported a positive effect of *Bacillus subtilis* IAA producing strains on the edible tubercle *Dioscorea rotundata* L in one of their

studies. They applied a suspension of *B. subtilis* on the surface of the plants, which resulted in an increase in stem and root length, increased fresh weight of the stem and root, an increase in the root:stem ratio and increased numbers of sprouts as compared with non-inoculated plants.

2.4.2. Regulating plant ethylene levels by rhizobacteria

Ethylene is essential for the growth and development of plants, but it has different effects on plant growth depending on its concentration in root tissues. At high concentrations, it can be harmful, as it induces defoliation and cellular processes that lead to inhibition of stem and root growth as well as premature senescence, all of which lead to reduced crop performance (Li et al. 2005). Under different types of environmental stress, such as cold, draught, flooding, infections with pathogens, presence of heavy metals, among others, plants respond by synthesizing aminocyclopropane, which is the precursor for ethylene (Chen et al. 2002; Glicket al. 2007). Some of the aminocyclopropane is secreted into the rhizosphere and is readsorbed by the roots, where it is converted into ethylene. This accumulation of ethylene leads to a downward spiral effect, as poor root growth leads to a diminished ability to acquire water and nutrients, which, in turn, leads to further stress. Thus, PGPR with the ability to degrade aminocyclopropane in the rhizosphere can help to break this downward cycle and reestablish a healthy root system that is needed to cope with environmental stress.

The primary mechanism that is used by rhizobacteria that degrade ethylene is the destruction of ethylene via the enzyme ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase, EC 4.1.99.4). This enzyme can diminish or prevent some of the

harmful effects of the high ethylene levels (Glick et al. 1998). The ACC deaminase acts on ACC (1-aminocyclopropane-1-carboxylate), an immediate ethylene precursor in higher plants, degrading this chemical to alpha-ketobutyrate and ammonium (Glick et al. 1998; Grichko and Glick 2001; Mayak et al. 2004). Rhizosphere bacteria with ACC deaminase activity belonging to the *Achromobacter* (Govindasamy et al. 2008), *Azospirillum* (Li et al. 2005), *Bacillus* (Ghosh et al. 2003), *Enterobacter* (Li et al. 2001), *Pseudomonas* (Govindasamy et al. 2008) and *Rhizobium* (Duan et al. 2009) genera have been isolated from different soils.

Various studies have demonstrated that plants treated with PGPR bacteria that produce ACC deaminase have increased their resistance to environmental stress. Grinchko and Glick (2001) inoculated tomato seeds with the ACC deaminase expressing bacteria *Enterobacter cloacae* and *Pseudomonas putida* and registered an increase in plant resistance on 55 days of age to 9 consecutive days of flooding. Ghosh et al. (2003) found ACC deaminase activity in three *Bacillus* species (*Bacillus circulans* DUC1, *Bacillus firmus* DUC2 and *Bacillus globisporus* DUC3), which stimulated root elongation of *Brassica campestri* plants. Mayak et al. (2004) evaluated tomato plants inoculated with the bacterium *Achromobacter piechaudii* under water and saline stress conditions. The authors reported a significant increase in fresh and dry weight of inoculated plants. In soils with a high copper content, Reed and Glick (2005) reported an increase in dry matter content of the root and the air part in raps seeds inoculated with the ACC deaminase producing bacterium *Pseudomonas asplenii*.

2.5. **Biocontrol of microorganisms causing plant disease**

Phytopathogenic microorganisms have a great impact on crop yields and can significantly reduce plant performance and crop quality. The usual strategy for the control of phytopathogens is to apply chemical pesticides, but this strategy has led to increased concerns over environmental contamination and has resulted in the so-called pesticide treadmill in which pathogens develop resistance to individual chemical controls over time, needing a constant development of new pesticides (Fernando et al. 2006). In this context, rhizobacteria that can provide biocontrol of disease or insect pests (biopesticides) are considered an alternative to chemical pesticides (Zahir et al. 2004). A large number of mechanisms are involved in biocontrol and can involve direct antagonism via production of antibiotics, siderophores, HCN, hydrolytic enzymes (quitinases, proteases, lipases, etc.), or indirect mechanisms in which the biocontrol organisms act as a probiotic by competing with the pathogen for a niche (infection and nutrient sites). Biocontrol can also be mediated by activation of the acquired systemic resistance (SAR), induced systemic resistance (ISR) responses in plants, and by modification of hormonal levels (Bowen and Rovira 1999; van Loon 2007) in the plant tissues.

2.5.1. Antibiotic-producing rhizobacteria

The production of antibiotics is considered one of the most powerful and studied biocontrol mechanisms for combating phytopathogens. Antibiotics constitute a wide and heterogeneous group of low molecular weight chemical organic compounds that are produced by a wide variety of microorganisms (Raaijmakers et al. 2002). Under

laboratory conditions many different types of antibiotics produced by PGPR have shown to be effective against phytopathogenic agents (Bowen and Rovira 1999). The antibiotics produced by PGPR include: butyrolactones, zwittermycin A, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, and 2,4-diacetyl phloroglucinol (DAPG) (Whipps 2001). The last is one of the most efficient antibiotics in the control of plant pathogens (Fernando et al. 2006) and can be produced by various strains of *Pseudomonas*, one of the most common bacterial species of the rhizosphere (Rezzonico et al. 2007). DAPG has a wide spectrum of properties in that it is antifungal (Loper and Gross 2007; Rezzonico et al. 2007), antibacterial (Cronin et al. 1997; Velusamy et al. 2006) and antihelmintic (Cronin et al. 1997). In soils, it suppresses the growth of the wheat pathogenic fungus *Gaeumannomyces graminis* var. *tritici*, Raaijmakers et al. (1999) reported a production of 0.62 ng DAPG per 10^5-10^7 CFU g^{-1} root by *P. fluorescens,* strain Q2-87.

2.5.2. Hydrogen cyanide (HCN) producing rhizobacteria

Apart from the production of 2,4 DAPG, some rhizobacteria are capable of producing HCN (hydrogen cyanide, also known as cyanide) (Rezzonico et al. 2007). HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and that also affects negatively the growth and development of plants (Siddiqui et al. 2006). HCN is a powerful inhibitor of many metal enzymes, especially copper containing cytochrome C oxidases. HCN is formed from glycine through the action of HCN synthetase enzyme, which is associated with the plasma membrane of certain rhizobacteria (reviewed by Blumer and Haas 2000). To date many different bacterial

genera have shown to be capable of producing HCN, including species of *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Devi et al. 2007; Ahmad et al. 2008). HCN production is a common trait within the group of *Pseudomonas* present in the rhizosphere, with some studies showing that about 50% of pseudomonads isolated from potato and wheat rhizosphere are able to produce HCN *in vitro* (Bakker and Schippers 1987; Schippers et al. 1990).

Various studies attribute a disease-protective effect to HCN, e.g. in the suppression of "root-knot" and black rot in tomato and tobacco root caused by the nematodes *Meloidogyne javanica* and *Thielaviopsis basicota*, respectively (Voisard et al. 1989; Siddiqui et al. 2006). The subterranean termite *Odontotermes obesus*, an important plague in agricultural and forestry crops in India, is also bio-controlled by HCN (Devi et al. 2007). However, there are investigations reporting harmful effects on plants, inhibition of energy metabolism of potato root cells (Bakker and Schippers 1987), and reduced root growth in lettuce (Alström and Burns 1989). Likewise, HCN produced by *Pseudomonas* in the rhizosphere inhibits the primary growth of roots in *Arabidopsis* due to the suppression of an auxin responsive gene (Rudrappa et al. 2008).

2.5.3. Siderophore-producing rhizobacteria

Siderophores are low molecular weight compounds that are produced and utilized by bacteria and fungi as iron (Fe) chelating agents. These compounds are produced by various types of bacteria in response to iron deficiency which normally occurs in neutral to alkaline pH soils, due to low iron solubility at elevated pH (Sharma and Johri 2003). Iron is essential for cellular growth and metabolism, such that Fe acquisition through siderophore production plays an essential role in determining the competitive fitness of

bacteria to colonize plant roots and to compete for iron with other microorganisms in the rhizosphere (see reviews: Crowley and Gries 1994; Crowley 2006). Siderophore producing PGPR can prevent the proliferation of pathogenic microorganisms by immobilising Fe^{3+} in the area around the root (Siddiqui 2006). Fe depletion in the rhizosphere does not affect the plant, as the low Fe concentrations occur at microsites of high microbial activity during establishment of the pathogen. Many plants can use various bacterial siderophores as iron sources, although the total concentrations are probably too low to contribute substantially to plant iron uptake. Plants also utilize their own mechanisms to acquire iron; dicots via a root membrane reductase protein that converts insoluble Fe^{3+} into the more soluble Fe^{2+} ion, or in the case of monocots by production of phytosiderophores (Crowley 2006). Various studies have isolated siderophore-producing bacteria belonging to the *Bradyrhizobium* (Khandelwal et al. 2002), *Pseudomonas* (Boopathi and Rao 1999), *Rhizobium* (Roy and Chakrabartty 2000), *Serratia* (Kuffner et al. 2008) and *Streptomyces* (Kuffner et al. 2008) genera from the rhizosphere.

Carrillo-Castañeda et al. (2002) reported positive effects on alfalfa plantlet growth after the inoculation of siderophore producing *Pseudomonas*, *Rhizobium* and *Azospirillum* grown in iron limited cultures. The inoculated alfalfa seeds increased their germination as well as the root and stem dry weight. Nevertheless, as with other PGPR, the growth promotion that occurred may be due to other mechanisms or combinations of mechanisms that increase nutrient availability, suppress pathogens, or affect root growth via hormone production.

2.6. **PGPR with multiple mechanisms of action**

The notion of multiple mechanisms emerged from early studies on *Azospirillum*, when the results of the field inoculation experiments failed to demonstrate that N-fixation was the main mechanism by which plant growth was stimulated (Bashan et al. 1989; Bashan and Levanony 1990). Failing N fixation as an explanation, the additive hypothesis was then proposed to describe the effect of *Azospirillum* on plant growth (Bashan and Levanony 1990). The additive hypothesis proposes the possibility of multiple mechanisms that function simultaneously or sequentially (Bashan and de-Bashan 2010). In the case of *Azospirillum*, nitrogen fixation has largely been discounted and primary plant growth promotion mechanism is now attributed to several other functions including phytohormones production (Dobbelaere et al. 1999; Malhotra and Srivastava 2008), ACC deaminase activity (Li et al. 2005) and hydrolytic enzyme production (Mostajeran et al. 2007). Today, it is increasingly recognized that many PGPR strain likely function by more than one mechanism (de Freitas et al. 1997). Examples of such bacteria are described in (Table 2.2) that refers to multiple mechanisms of action for various PGPR isolates (Vassilev et al. 2006; Ahmad et al. 2008; Avis et al. 2008).

Two recent reviews reconsider the importance of the presence of multiple action mechanisms promoting plant growth in the microorganisms. Vassilev et al. (2006) reviewed the potential of phosphorus solubilizing microorganisms that can provide simultaneously phytopathogen biocontrol, and also affect plant growth via production of siderophores, hydrolytical enzymes and IAA. A second review by Avis et al. (2008) classified PGPR bacteria into two groups based on the main action mechanisms by which they are known. These two groups are: (i) microorganisms with direct plant growth promoting mechanisms (e.g. phytohormone production, phosphorus

solubilization, etc.), and (ii) microorganisms which indirectly promote plant growth and productivity through biocontrol of phytopathogens (e.g. production of siderophores, antibiotics, HCN, etc.). Despite this arbitrary classification, microorganisms of both groups can simultaneously contain secondary mechanisms.

2.7. **Practical considerations in the use of PGPR**

Studies on the use of PGPR inoculants have been conducted under laboratory (soil microcosms), greenhouse and field conditions (Table 2.3), but they can often lead to inconsistent results when compared under different experimental conditions. Many experiments have demonstrated the growth stimulation of plant crops in the greenhouse, resulting in increased yield parameters and in the control on soil-borne pathogenic organisms. However, the replication of successful results of PGPR applications under field conditions has been limited by the lack of knowledge about their ecology, survival and activity in the plant rhizosphere. The main aspects related to the application of bacterial inoculants are discussed below.

2.7.1. Efficacy of PGPR inoculation

PGPR efficacy is dependent on establishing an effective population density of active cells in plant rhizosphere. As this is a simple principle, it has proved to be difficult to establish dose response effects in which the degree of plant growth promotion or disease suppression can be directly correlated with size of the PGPR population. In general, bacterial suspensions of PGPR are prepared at densities of 10^8 to 10^9 CFU ml^{-1} for root dipping and soil inoculation. After inoculation at these high densities, the cell numbers

will undergo a rapid decline depending on whether or not the soil has been sterilized. In autoclaved soils, inoculants will typically persist at cell densities of 10^7 to 10^8 CFU g^{-1} soil for many weeks. In nonsterile soils where there is competition with the resident flora and predation by protozoa and nematodes, bacterial populations will decline rapidly by orders of magnitude per week until the population reaches equilibrium with its environment. This likely accounts for differences that are observed in lab and greenhouse studies where soils are sterilized, versus in the field where results of PGPR inoculation are much more inconsistent.

Inoculation efficacy depends on the rhizosphere competence of the bacteria for the particular host plant. In studies examining the induction of systemic induced resistance to foliar and systemic pathogens, model systems with cucumber, carnation, and bean show that effective root colonization levels can be achieved by seed coating with high numbers of bacteria or by use of bacteria suspensions to dip the plant roots or inoculate the soil at the time of transplanting (Zehnder et al. 2001). To maintain effective cell densities under field conditions, it is often necessary to reinoculate at intervals during the production period. The latter strategy is limited by the high cost of inoculum production, difficulty in maintaining viable cells for long periods of time in storage, and cost for delivery and incorporation of inoculants into the field. So far, the most commercially successful inoculants have been gram positive spore forming bacteria, which can persist in storage from months to years, and that can withstand starvation, temperature, moisture and other environmental stresses better than nonspore forming bacteria.

Table 2.2. Promoting growth plant rhizobacteria strains with multiple mechanisms.

Species	CP	IAA	ACCD	HCN	SID	Other mechanisms	Reference
Azotobacter chroococcum A4	+	+	ND	+	+		Wani et al. 2007
Bacillus sp. PSB1	+	+	ND	+	+		Wani et al. 2007
Bacillus sp. PSB10	+	+	ND	+	+		Wani et al. 2007
Enterobacter BNM 0357	+	+	ND	ND	ND	Nitrogenase +	Shoebitz et al. 2009
Enterobacter sp. NBRI K28	+	+	+	ND	+		Kumar et al. 2008
Pseudomonas sp. SF4c	-	+	-	ND	+		Fischer et al. 2007
Pseudomonas aeruginosa BFPB9	+	+	ND	+	+	Protease+, Cellulase+	Jha et al. 2009
P. fluorescens PSRB21	+	+	ND	ND	+		Hariprasad and Niranjana 2009
P. mosselli FP13	+	+	ND	-	+	Protease+	Jha et al. 2009
P. fluorescens biotype G (N3)	ND	+	+	ND	ND	Chitinase+	Shaharoona et al. 2006
P. plecoglossicida FP12	+	+	ND	-	+	Protease+	Jha et al. 2009
P. putida PSRB6	+	+	ND	ND	+	Chitinase+	Hariprasad and Niranjana 2009
Serratia marcescens	ND	+	ND	ND	+		Kuffner et al. 2008

CP: tricalcic phosphate solubilization; IAA: Indol acetic acid production; ACCD: ACC deaminase activity; HCN: Hydrogen cyanide production; SID: Siderophore production; + positive, - negative, ND: not done.

Table 2.3. Plant crop response to PGPR inoculation under different experimental conditions.

Plant	PGPR inoculant	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Apple (*Malus domestica* L.)	*Bacillus* M3, *Bacillus* OSU-142 and *Microbacterium* FS01	N-fixing and phosphate solubilizing	- Cumulative yield (kg tree^{-1}) - Average fruit weight (g) - Average fruit diameter (mm) - Total soluble solid (%) - Average shoot length (cm) - Average shoot diameter (mm) - P contents of leaves (%)	26–88 14–25 2–15 -6–2 16–29 16–18 3–45	- Field experiment - Non commercial strains	Karlidag et al. 2007
Cotton (*Gossypium* sp.)	*Bacillus subtilis* FZB 24®	IAA production, phytase activity and antibiotics production	- Average yield (t ha^{-1}) - Bolls/plant mean number - Mean plant height cm	31 19 11	- Field experiment - Commercial strain	Yao et al. 2006
Maize (*Zea mays* L.)	*Azotobacter*	IAA production	- Straw yield (t ha^{-1}) - Fresh biomass (t ha^{-1}) - Plant height (cm) - Fresh cob weight (g) - Cob length (cm) - Grain rows cob^{-1} - 1000-grain weight (g)	17 12 7 13 6 3 7	- Field experiment - Non commercial strains	Zahir et al. 2005

[a]Percentage increase over non-inoculated control

Continued…

Plant	PGPR inoculant	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Maize (*Zea mays* L.)	*Pseudomonas fluorescens* (MPp4), *Burkholderia* sp. (MBp1, MBf21 and MBf15)	IAA production and antagonism against *Fusarium verticillioides*	- Shoot length (cm) - Longest root length (cm) - Shoot fresh weight (g) - Root fresh weight (g) - Plants showing disease symptoms (%) - Disease reduction (%)	30–32 47–63 24–32 76–88 10–30 60–87	- Microcosm and greenhouse experiments - Non commercial strains - Not proven at field level	Hernández-Rodríguez et al. 2008
Oat (*Avena sativa* L)	*Azospirillum* sp. (ChO6 and ChO8) *Azotobacter* sp.(ChO5) *Pseudomonas* sp. (ChO9)	IAA production and acetylene reducing activity	- Root length (mm) - Root area (cm^2) - Shoot dry weight (mg $plant^{-1}$) - Total N (mg $plant^{-1}$) - Proportion of plant N fixed from the atmosphere (% Ndfa)	-12–23 8–500 6–93 -50–50 50–64	- In vitro - Non commercial strains - Not proven at field level.	Yao et al. 2008
Raspberry cv Heritage (*Rubus* spp)	*Bacillus* M3	N-fixing and phosphate solubilizing	- Cane length (cm) - Cane diameter (mm) - Number of picks - Number of cluster - Number of berries - Leaf area (cm^2) - Total soluble solid (%) - Titratable acids (%)	13 6 -3 25 25 14 -0.9 2	- Field experiment - Non commercial strains	Orhan et al. 2006

[a]Percentage increase over non-inoculated control

Continued…

Plant	PGPR inoculant	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Red pepper cv Barodda (*Capsicum annuum* L.)	*Azospirillum brasilense* CW903, *Burkholderia pyrrocinia* CBPB-HOD, *Methylobacterium oryzae* CBMB20	IAA production, P solubilizing and N fixing	- Shoot length (cm) - Root length (cm)	4–35 0.4–17	- Greenhouse experiment - Non commercial strains - Not proven at field level.	Madhaiyan et al. 2010
Rice cv. Dongjin (*Oryza sativa* L.)	*Azospirillum brasilense* CW903, *Burkholderia pyrrocinia* CBPB-HOD, *Methylobacterium oryzae* CBMB20	IAA production, P solubilizing and N fixing	- Shoot length (cm) - Root length (cm)	1.5–8.5 20–31	- Greenhouse experiment - Non commercial strains - Not proven at field level.	Madhaiyan et al. 2010
Sorghum (*Sorghum bicolour* (L.) Moench)	*B. cereus (*KBE7-8*)* *B. cereus (*NAS4-3*) and Stenotrophomonas maltophilia (*KBS9-B)	Siderophore production, IAA production and phosphate solubilization	- Shoot height (mm) - Shoot fresh weight (g) - Shoot dry weight (g) - Chlorophyll (spad units) - Leaf width (mm) - Root length (mm) - Root dry weight (g)	104–182 1133–2255 180–260 68–78 103–326 214–279 1300–1525	- Greenhouse pot trial - Non commercial strains - Not proven at field level.	Idris et al. 2009

[a]Percentage increase over non-inoculated control

Continued…

Plant	PGPR inoculant	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Sweet cherry cv. 0900 Ziraat (*Prunus avium* L.)	*Pseudomonas* BA-8 and *Bacillus* OSU-142		- Yield per trunk cross-sectional area (kg cm^{-2}) - Fruit weight (g) - Fruit diameter (mm) - Total soluble solid (%) - Titretable acidity (%) - Shoot length (cm) - Shoot diameter (mm)	11–22 1–5 0.2–1 1–4 -0.4–3 11–29 -0.5–0.7	- Field experiments - Non commercial strains	Esitken et al. 2006
Tomato cv Rio Fuego (*Lycopersicon esculentum* Mill)	*Bacillus subtilis* BEB-lSbs (BS13).		- Yield $plant^{-1}$ (g) - Marketable grade yield (%) - Weight/fruit (g) - Length (cm) - Diameter (cm)	21–25 6–20 18–29 9–18 4–5	- Greenhouse experiments - Non commercial strains - Not proven at field level.	Mena-Violante and Olalde-Portugal 2007
Tomato cv Mairoku (*L. esculentum* Mill.)	*Azospirillum brasilense* CW903, *Burkholderia pyrrocinia* CBPB-HOD, *Methylobacterium oryzae* CBMB20	IAA production, P solubilizing and N fixing	- Shoot length (cm) - Root length (cm) - Stem girth (mm)	8–13 1–13 5–11	- Greenhouse experiment - Non commercial strains - Not proven at field level.	Madhaiyan et al. 2010

[a]Percentage increase over non-inoculated control

Methods for inoculation with either gram negative or gram positive PGPR bacteria require the use of a carrier to deliver the inoculum into the soil and allow mixing of the cells in the soil profile. This can involve low cost carriers such as peat, calcined clay, or powdered corn cobs that are mixed with the bacterial suspensions and dried. Alginate microbeads are also used and provide many advantages by incorporating the cells into a protected matrix that undergoes decomposition in the soil and slowly releases the bacteria. Lastly, bacteria can be introduced into the irrigation water via on-site fermentation equipment that automatically cultures the bacteria and pumps them into the irrigation water at desired intervals. While not widely used, studies employing this technology have shown that it is possible to maintain effective cell densities of pseudomonads in a citrus orchard for control of root rot caused by *Phytophthora cinammomi* (Steddom et al. 2002) over the whole year. Equipment for irrigation based inoculum delivery continues to improve and provides an innovative method for assuring high cell densities of PGPR, with particular advantages for allowing utilization of gram negative bacteria as soil inoculants.

PGPR population densities are typically much higher in the plant rhizosphere than in the bulk soil. However, correlating population density to activity is a great challenge. The rhizosphere is very heterogenous with respect to nutrient availability. Mature roots are typically colonized by bacteria at densities of 10^8 to 10^9 CFU g^{-1} in the mature root zones. However, there is extreme competition between bacteria on the mature roots where the community is crowded with oligotrophic bacteria that are selected based on their ability to use recalcitrant materials such as cellulose and lignin and their ability to grow at very slow rates. In contrast, total bacterial cell densities are orders of magnitude lower in the apical root zones and in the zone of elongation behind the root tips, but the cells are more active due to the localized release of root exudates.

Thus, the population size, PGPR activity and expression of relevant genes will vary in different root locations. To be effective, PGPR used for biocontrol of root disease must be active in the same location as the pathogen.

A number of approaches are commonly used to quantify PGPR, including measurements of cell densities based on 16S rRNA gene copy numbers or plating on agar and CFU enumeration. Other approaches employ PCR methods to quantify the copy number of a particular functional gene, or expression of relevant mRNA for genes encoding PGPR traits. A common problem in much research on PGPR has been the failure to monitor the cell density of the introduced bacteria over time to confirm that inoculation was effective. In such cases, it is not possible to determine whether PGPR are responsible for the observed effects or to explain variations in efficacy of the inoculants that may be caused by management or environmental factors.

Mathematical modeling of the behavior of PGPR soil inoculants has been used to predict how various environmental factors affect the survival and activity of PGPR soil inoculants (Strigul and Kravchenko 2006). Supporting much experimental work, the model by Strigul and Kravchenko illustrates that survival and growth of newly introduced bacteria are strongly limited by competition for organic substrates with the resident microflora. PGPR are predicted to be the most effective in soils with low organic matter or stressed soils where growth of the indigenous population is restricted. In the case of disease suppressive pseudomonads that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), the effective population size to obtain suppression of take-all decline of wheat caused by *Guamannomyces graminis* is in the range of 10^5 to 10^6 CFU gram soil^{-1}. Effective isolates with good rhizosphere competence can be added to the soil at 10^4 CFU and will grow to densities of at least 10^5 CFU g root^{-1} (Bankhead et al. 2004). Interestingly, disease suppression effects occur at this threshold

cell density and are not enhanced at higher cell densities (Raaijmakers and Weller 1998). This suggests that once a critical cell mass has been achieved, there is a quorum mediated signal that results in expression of antibiotic production at concentrations that provide the biocontrol.

To date, there is very limited knowledge of how specific inoculants interact with resident microbial populations (Haas and Keel 2003). Nonetheless, many resident bacteria, possibly including nonculturable bacteria, will carry genes encoding common PGPR functions. In the case of quorum regulated genes, such as those for antibiotic and siderophore production, there is a broad intra and interspecific communication level between different bacterial populations. Thus, it may result in either positive and negative feedback on quorum sensor mediated behavior (Pierson et al. 1998). Prior experiments examining the effects of wheat inoculation with 2,4-DAPG producing pseudomonads have shown that there are broad interaction levels not only with various genotypes of resident fluorescent pseudomonads, but with populations of diverse bacterial species including *Arthrobacter, Chyrseobacterium, Flavobacteria*, and other species that are significantly enriched in the presence of 2,4-DAPG producers (Landa et al. 2003). Similarly, a recent study by Roesti et al. (2006) showed striking shifts in rhizobacterial community structures following inoculation with various combinations of PGPR pseudomonads. However, such interactions are variable and even strain specific for different inoculants. In a study comparing three pseudomonads, relatively minimal changes in community structure of the rhizosphere occurred on wheat grown over multiple cycles (Bankhead et al. 2004), but each inoculant shifted the community in a distinct manner. A larger question is whether inoculation can result in shifts in community structure that increase plant growth promotion and disease suppression functions of the resident community. This question will only be answered once

molecular tools are available to detect and quantify all the PGPR relevant phenotypes in the microbial community. As most bacteria in the rhizosphere are still uncultured, this will require a metagenomics approach to identify the genes. Quantitative PCR arrays or DNA microarrays will also provide valuable tools for examining the response patterns of microbial communities to soil inoculants.

2.7.2. Potential marker genes for PGPR monitoring at field level

Potential marker genes for PGPR functions include those encoding enzymes for antibiotic production, hydrogen cyanide, ethylene destruction and auxin promotion. HCN genes are broadly distributed among many 2,4-DAPG producing pseudomonad strains (Haas and Défago 2005). PCR primers for a conserved sequence in the *hcnAB* genes have been shown to be specific for detection of HCN producing pseudomonads from a world-wide collection of isolates (Svercel et al. 2007). Similarly, primers are available to target production of 2,4-DAPG (Bergsma-Vlami et al. 2005). Typically these primers have been used to test isolates that are cultivated from soil, but can be used with soil with the caveat that gene products that are produced. The PCR products should be further analyzed by DNA melting and size check (570 bp fragment) and sequencing to determine the primers have not amplified other genes. An advantage with these primers is that the forward primer can be combined with a GC clamp for analysis of the PCR products by DGGE to assess the genotypic diversity of indigenous DAPG-producing *Pseudomonas* isolates.

Another target gene for quantification is the *accA* gene that encodes aminocyclopropane carboxylic acid deaminase. The *accA* gene is broadly distributed among a wide range of Gram negative bacteria including PGPR pseudomonads, and is

common in many Gram-positive bacteria, rhizobia, and fungi (see review: Glick et al. 2007). Here again, caution must be used during interpretation of changes in *accA* gene copy numbers, as some bacteria have sequences that are highly similar, but they code instead for enzymes with other functions such as serine deamination. Using PCR methods to detect and quantify PGPR target genes in fallow soils, our experience has been that cell numbers of PGPR and copy numbers of PGPR relevant genes may fall below detection limits, such that the populations are better estimated by culture of a host plant in the soil and baiting of the populations on to the roots where they can readily be quantified in their working habitat.

2.8. **Concluding remarks and future trends**

The use of PGPR inoculants to improve agricultural production has been demonstrated in numerous studies and the basic mechanisms are now well understood. PGPR, in accordance with their mode of action, can be classified as biofertilizers, phytostimulators and biopesticides, with certain bacteria having overlapping applications. It is becoming increasingly apparent that most PGPR can promote plant growth by several mechanisms, but most studies currently focus on individual mechanisms and have not been able yet to sort out the relative contributions of different processes that are responsible for plant growth promotion. Screening strategies for selecting the best strains will require more comprehensive knowledge of the traits required for rhizosphere competence, and studies on the ecology of introduced PGPR with the resident PGPR and other microbial species in the plant rhizosphere. While inoculation is now viewed as a means to enhance plant growth, the effects of various management practices or soil amendments on PGPR activity of indigenous bacteria

remain unknown. The use of PGPR inoculants in agriculture is already proceeding, and offers many opportunities to improve plant nutrition, crop yields, and disease management, while improving sustainability by reducing the need for chemical inputs. Nevertheless, as our understanding of the ecology of these bacteria improves, it should be possible to obtain a more informed explanation of the mechanisms that are involved in plant growth promotion and identify situations in which bioaugmentation with soil inoculants may be useful for increasing crop yields.

2.9. **References**

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Chapter 3

Influence of Nitrogen Fertilisation on Pasture Culturable Rhizobacteria Occurrence and the Role of Environmental Factors on Their Potential PGPR Activities

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Abstract

Plant growth-promoting rhizobacteria (PGPR) naturally occur in the rhizospheres of pasture, but still little is understood regarding how soil agricultural practices affect them. Here, we examined the effects of long-term nitrogen (N) fertilisation on the occurrence of potential culturable PGPR in rhizosphere soils from pastures grown in Chilean Andisols. We also evaluated *in vitro* the effects of organic acids (citric, malic and oxalic acids), metals (Al and Mn), and N supply (urea and ammonium sulphate) on indole acetic acid (IAA) production and phosphorus (P) liberation by selected strains. Compared with non-N-fertilised pasture, N fertilisation significant increased (30%) the occurrence of culturable phosphobacteria, but decreased (7%) the occurrence of IAA-producing rhizobacteria. Most efficient IAA-producing phosphobacteria (IAAP) were identified as *Bacillus*, *Enterobacter*, *Pseudomonas* and *Serratia*. At low pH (4.8), the assays showed that the IAA production by *Serratia* sp. N0-10LB was increased (31-74%) by organic acids. On the other hand, the IAA production by *Pseudomonas* sp. N1-55PA was increased 2-5 fold by metals. In all strains, the growth and IAA production were significant decreased by 500 μM of Al, except *Serratia* sp. N0-10LB, suggesting its potential as PGPR for Chilean Andisols. When urea was added as main N source the bacterial growth and P utilization significant increased compared with ammonium sulphate. The influence of environmental factors that are typical of Chilean Andisols on rhizobacterial communities will provide better management practices to enhance their PGPR functions as well as a better selection biofertilisers to be used in Chilean Andisols.

Keywords: indole acetic acid; nitrogen fertilisation; aluminium; phosphobacteria; volcanic soils

3.1. **Introduction**

Plant growth-promoting rhizobacteria (PGPR) occur in all soils and enhance plant health and productivity by improving nutrient availability, phytostimulator production, and disease suppression. While there is considerable interest in the development of PGPR as biofertilisers that can be used to augment soil microbial communities, there has been relatively little research to examine the influence of agricultural management practices and environmental factors on PGPR activities of indigenous bacteria (Evans et al. 1993; Malboobi et al. 2009).

Agricultural production in southern Chile is based on ash-derived volcanic Andisols, which are characterized by low pH (4.8-5.8) and high content of organic matter and total phosphorus. Cultivated Andisols present high amounts of total P (1, 422 - 4, 011 mg kg^{-1}), organic P (870 - 3,197 mg kg^{-1}) and low available P (Borie and Rubio 2003). In these soils, the acidification results from the use of urea and other ammonia fertilisers (Mora et al. 2005, 2007). In Chile, there is an increasing need to improve the management of grazing systems and to reduce both the negative impact of N leaching and the chemical reaction in acid soils. The use of large amount of N supplied as urea is the most common practice in milk and meat production system based on pastures (Nuñez et al. 2010a, 2010b). In these systems, the nitrification process contributes to soil acidification because of free H^+ release. Due to the acid condition, which characterizes volcanic soils, acidic reaction of fertilisers applied continuously to soil overcomes their buffer capacity and the acidification process is accelerated. About 50 % of these soils present a high soil acidity level, the main factor limiting pasture production (Mora et al. 1999, 2002, 2006).

P solubilisation (PS), P mineralisation (PM) and indole acetic acid (IAA) production are desirable traits in PGPR. Phosphate-solubilising/mineralising rhizobacteria, also known as phosphobacteria, are common components of soils, and they are well known to enhance the phosphorus (P) availability for plant growth (Unno et al.2005; Collavino et al. 2010). On the other hand, IAA-producing rhizobacteria have been reported to significantly enhance the development of the host plant root system, which may improve mineral uptake, particularly P (Marschner et al. 2011). In general terms, it is known that some environmental factors, such as pH, may affect soil bacterial diversity (Fierer and Jackson 2006). In soils with low pH, investigations has been reported that member of family *Enterobacteriaceae* are the predominant group within phosphate solubilizing rhizobacteria populations (Pérez et al. 2007; Collavino et al. 2010). Several studies have also revealed that the PS and IAA production by rhizobacterial isolates may be modulated by pH (Nautiyal et al. 2000; Malboobi et al. 2009), temperature (Nautiyal et al. 2000; Ona et al. 2005; Malboobi et al. 2009), and nutrient availability (Gyaneshwar et al. 1999; Jorquera et al. 2008; Malhotra and Srivastava 2009).

In Chilean Andisol, soil acidity also increases the concentration of metal cations in soil solution, such as aluminium (Al^{3+}) and manganese (Mn^{2+}), which can be toxic for plant growth (Mora et al. 2006; Millaleo et al. 2010). In response to the presence of toxic metals, plants often respond by increased exudation of organic acids and Al-phosphate complexes as a tolerance mechanisms (Borie and Rubio 2003; Mora et al. 2009). Also the high P fixation at low pH and the high organic P in Andisols make necessary to develop other alternative management practices such as the use of biofertiliser. In this context, we have isolated phosphobacteria from plants grown in Andisol, which are now being studied as potential soil inoculants to increase P

availability in Chilean Andisols (Jorquera et al. 2008, 2011). We also have detected the presence of mobile genetic traits related to Al tolerance in rhizobacteria populations associated with pasture and cereal plants grown in Andisols (Jorquera et al. 2010). Thus, the development of microbial inoculants to increase the P nutrition of plant grown in Chilean Andisols must consider its efficiency under urea fertilisation, low pH and presence of metals. The objectives of this research were : 1) to evaluate the effect of long-term nitrogen fertilisation on the occurrence of potential culturable PGPR in pastures grown Chilean Andisol, 2) to evaluate in vitro the effects of organic acids (citric, malic and oxalic acids), metals (aluminium [Al] and manganese [Mn]), and N supply (ammonium sulphate and urea) on the potential PGPR activities (indole acetic acid [IAA] production and P liberation) of selected IAA-producing phosphobacteria (IAAP).

3.2. **Materials and Methods**

Soil sampling

Rhizospheric soil samples of were collected from pastures located in Maquehue Experimental Station (38°50′ S and 72°41′ W). The pastures are managed by La Frontera University and have a history of fertilisation with triple super phosphate (400 kg ha^{-1} yr^{-1}) and potassium magnesium sulphate (300 kg ha^{-1} yr^{-1}). The pastures were initially established in 2004 with perennial ryegrass (*Lolium perenne* cultivar Aries) and divided in plots without (N0) and with (N1) a fractionated long-term urea fertilisation (equivalent to 600 kg N ha^{-1} yr^{-1}). The absence of N supply in N0 have resulted in changes in botanical composition (68% ryegrass and 32% resident species) compared with N1 (100% ryegrass). In contrast, the application of N in soils has produced a continuous decrease of pH (Fig. 3.1), with the concomitant increase of Al saturation (Mora et al. 2002, 2006), but an increase of accumulated pasture production for season (14.6 Mg ha^{-1}) compared with unfertilized pasture (2.2 Mg ha^{-1}).

Rhizospheric soil samples (including roots and adhering soil) were randomly collected from each plots (N0 and N1). The samples were collected in triplicate (0−10 cm) by with a cleaned spade, stored in coolers at 4ºC and transported to the laboratory within a few hours where they were immediately processed.

Occurrence of potential plant growth-promoting rhizobacteria

The culturable rhizobacteria were isolated using four types of general agar media as follows: Luria-Bertani agar (LB), nutrient agar (NA), *Pseudomonas* agar base (PAB)

and R2A agar (R2A). After the incubation period, bacterial colonies differentiated by morphology, pigmentation and growth rate were randomly selected. Approximately 50 different colonies were isolated from each type of agar media separately inoculated with three samples from each plot. The screening for culturable phosphobacteria was carried out on National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal 1999) and phytase-screening medium (PSM) (Kerovuo et al. 1998), respectively. After a 4-d incubation, halo formations around the colonies indicated bacterial PS and PM activities. The isolates were selected on the basis of a halo zone produced in the NBRIP/PSM agar.

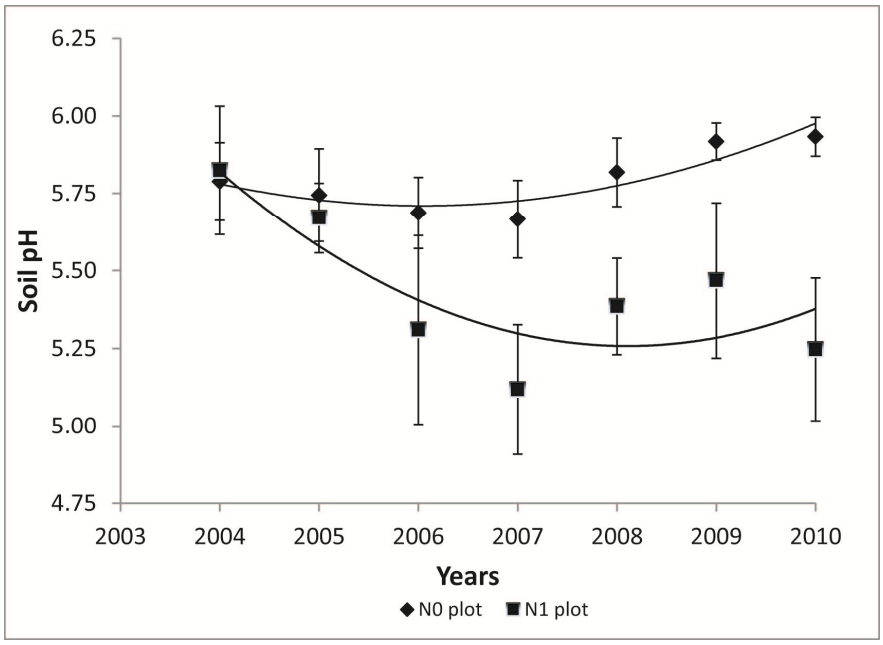

Figure 3.1. Effect of long-term N fertilizer application on soil pH of pastures. Bars represent mean ± standard deviation (n = 6)

The culturable rhizobacteria with ability to produce indole acetic acid (IAA) were detected in supplemented LB broth according to standard methods previously described by Patten and Glick (2002) with the modification of adding 5 mM L-tryptophan to the LB broth. The qualitative detection of produced IAA was carried out

in microplates (96-well plates), and the quantitative estimation was carried out in 15-ml tubes. The quantitative determination was accomplished with the following parameters that simulated the rhizosphere of acidic volcanic soils: 10-fold dilution of LB broth with a pH of 4.8. After incubation (48 h at 30°C with shaking), bacterial cells were removed from the culture medium by centrifugation at 10,000 *g* for 10 min. The supernatant (1 ml) was mixed with 2 ml of Salkowski's reagent, and the mixture was incubated at room temperature for 30 min. The appearance of a red colour indicated IAA production. The colour intensity was determined at 530 nm using a spectrophotometer (Metertek SP-830). The concentration of IAA was determined by a standard curve prepared with known concentrations of IAA (Sigma-Aldrich). The amount of IAA produced was expressed as $\mu g\ ml^{-1}$. Three independent replicates of each isolate were analysed.

Genetic and enzymatic characterisation of indole acetic acid-producing phosphobacteria

Based on screening of potential PGPR, ten indole acetic acid-producing phosphobacteria (IAAP) were selected from each plot on the basis of the halo zone (width > 3 mm) produced in NBRIP/PSM agar and IAA production (> 5 $\mu g\ ml^{-1}$). The selected IAAP strains were then characterised according to the partial sequencing of 16S rRNA gene. The 16S rRNA gene characterisation procedure was carried out as previously described by Jorquera et al. (2008). Briefly, genomic DNA was extracted from bacterial cultures grown in LB broth overnight at 30ºC. Amplification of the variable region of the 16S rDNA gene was performed by polymerase chain reaction (PCR) using primers previously described by Muyzer et al. (1998). The 358F (CCT ACG GGA GGC AGC AG) and 907R (CCG TCA ATT CMT TTG AGT TT) primers were used to amplify the V3-V4 variable region, which allowed characterisation at the

genus level for each isolate. The 16S rDNA fragments were sequenced by Macrogen (Seoul, Korea). The obtained DNA sequences were compared with sequences in the GenBank database using BLAST tools. The nucleotide sequences generated in this study were deposited in the GenBank.

Effect of organic acids and metals on the production of indole acetic acid by selected rhizobacteria

The two most efficient IAAP were chosen from each pasture on the basis of their ability to produce IAA (15-60 µg ml^{-1}). These strains were grown in 10-fold diluted LB broth supplemented with organic acids (citric, malic and oxalic acid) and toxic metals (aluminium and manganese). The samples of culture media were inoculated with approximately 5 x 10^5 cells ml^{-1}, and the cultures were incubated for 48 h at 30°C with shaking. The same procedure was followed for the quantitative determination of IAA and the bacterial growth was measured by optical density at 600 nm (OD_{600}). The results were expressed as a ration between IAA production and bacterial growth.

The concentrations assayed of each organic acid were 10, 50 and 100 µM and to metals, the following concentrations were used: 50, 200 and 500 µM for Al and 50, 100 and 350 µM for Mn. All the concentrations assayed are according to values reported in the literature for rhizosphere (Jones 1998, 2003; Igual and Dawson 1999; Li et al. 2002) soils and values obtained by our research group in Andisols (Rosas et al. 2007; Mora et al. 2009).

Effect of N on the release of phosphate by selected rhizobacteria

The effect of N on PS ability of rhizobacteria was tested by growing the strains in NBRIP broth containing three concentrations (7.5, 15 and 30 mM) of ammonium sulphate and urea. These concentrations are those suggested by Cartes et al. (2009) for Chilean Andisols. The quantitative estimation of PS activity in NBRIP broth was carried out using 15-ml tubes containing 5 ml of medium inoculated in triplicate with the selected bacterial strains. Uninoculated media served as blank. The tubes were incubated at 30ºC for 48 h. The bacterial growth was measured by the quantification of total protein content in microbial biomass by Bradford (Kruger 2002).

The liberated P from the NBRIP broth was measured according to the ammonium molybdate method as previously described by Greiner (2004) with slight modifications. After incubating the samples for 48 h at 30ºC, 1 ml of the culture medium was centrifuged at 9,300 *g*, and 20 µl of the supernatant was added to 270 µlof distilled water, 1150 µl of solution A (acetone, 5 N H_2SO_4 and 10 mM ammonium molybdate; 2:1:1 v/v) and 80 µl of citric acid. The inorganic P liberation capacity was determined by spectrometry at 355 nm.

Data analysis

The data were analysed by a one-way analysis of variance (ANOVA), and comparisons were carried out for each pair with Student's t-test using JMP statistical software (version 5.0; SAS Institute, Inc.). All experiments were carried out in triplicate, and the values are given as means ± standard errors. Differences were considered to be significant when the *P-value* was less than or equal to 0.05.

3.3. **Results**

Occurrence of potential plant growth-promoting rhizobacteria

A total of 1,176 bacterial strains were isolated from the rhizosphere of the perennial ryegrass. Of these strains, 620 strains were derived from N0, and 556 strains were derived from N1. Table 3.1 shows the percentage of culturable rhizobacteria that showed plant growth-promoting traits. A significant ($P \leq 0.05$) higher percentage of culturable rhizobacteria that solubilise phosphate on NBRIP and mineralise phytate on PSM only (PS + PM) was found in N1 (47%) as compared with N0 (14%) (Table 3.1). In contrast, a significant ($P \leq 0.05$) higher percentage of culturable rhizobacteria that with the ability to produce IAA only were found in N0 (10%) compared with N1 (3%).

According to the analysed mechanisms, approximately 10-13% of the culturable phosphobacteria also showed the ability to produce IAA (IAAP). The occurrence of culturable rhizobacteria without any mechanisms was also found, and the percentage of these bacteria was higher in N0 (31%) than with N1 (11%).

Characterisation of indole acetic acid-producing phosphobacteria

The analyses of 16S rRNA gene sequences of 20 selected IAAP strains revealed the presence of rhizobacteria belonging to the following taxa: *Enterobacteria*, *Bacillus*, *Flavobacteria*, *Pseudomonas* and *Serratia* (Table 3.2). It is noteworthy that all the IAAP isolated from non-N-fertilised pastures (N0) were *Enterobacteria*.

Effect of organic acids and metals on the production of indole acetic acid by selected rhizobacteria

The effect of organic acids on IAA production by most efficient IAAP (*Bacillus* N1-19NA, *Enterobacter* N0-29PA, *Pseudomonas* N1-55PA and *Serratia* N0-10LB) strains is shown in the Figure 3.2. The IAA production by *Bacillus* N1-19NA and *Pseudomonas* sp. N1-55PA strains were not affected when the strain was incubated in LB broth supplemented with the three organic acids at the three concentrations tested. With *Enterobacter* N0-29 PA strain, the addition of three organic acids significant ($P \leq 0.05$) decreased (34-50%) the production of IAA. In contrast, the IAA production by *Serratia* sp. N0-10LB was significant ($P \leq 0.05$) increased (31-74%) with three organic acids.

The Figure 3.3 shows the effect of metals on IAA production by most efficient IAAP strains. The IAA production of the *Pseudomonas* N1-55PA was significant ($P \leq 0.05$) increased 2-5 fold by addition of Al and Mn at the two concentrations tested. The concentration of Al 500 μM affected the growth of IAAP, except *Serratia* N0-10LB which showed similar IAA production compared with control without Al.

Effect of N on the release of phosphate by selected rhizobacteria

The phosphate liberation by efficient IAAP strains showed that the N compounds type used as main N source, affect significantly the bacterial growth and phosphate content in liquid medium (Fig. 3.4). Higher bacterial growth was observed when the broth was supplemented with urea compared with ammonium sulphate. However, the level of phosphates found in broth in the presence of urea was lower for all strains.

Table 3.1. Percentages (%) of culturable rhizobacteria with potential PGPR traits isolated from the rhizosphere of non-N-fertilised (N0) and N-fertilised (N1) pastures plots.

Potential PGPR traits	N0	N1
PS	3.6[a]	3.3[b]
PM	19	16.3
IAA	10.4*	3
PS+PM	14.3	46.5*
PS+IAA	0.8	0.2
PM+IAA	10.2	6.1
PS+PM+IAA	10.5	13.1
None[§]	31.2*	11.5

[a]Percent in relation to 600 colonies randomly chosen from general media agar (LB, NA, PAB and R2A). [b]Percent in relation to 556 colonies randomly chosen from general media agar (LB, NA, PAB and R2A). PS: culturable rhizobacteria that solubilise phosphate on NBRIP only; PM: culturable rhizobacteria that mineralise phytate on PSM only; IAA: culturable rhizobacteria that produce indole acetic acid in LB broth only. PS+PM: culturable rhizobacteria that solubilise phosphate on NBRIP and mineralise phytate on PSM only. PS+IAA: culturable rhizobacteria that solubilise phosphate on NBRIP and produce indole acetic acid in LB broth only; PM+IAA: culturable rhizobacteria that mineralise phytate on PSM and produce indole acetic acid in LB broth only; PS+PM+IAA: culturable rhizobacteria that solubilise phosphate on NBRIP, mineralise phytate on PSM and produce indole acetic acid in LB broth. [§]culturable rhizobacteria that do not solubilise phosphate on NBRIP, do not mineralise phytate on PSM and do not produce indole acetic acid in LB broth.* denote significant difference ($P \leq 0.05$).

In assays with *Bacillus* N1-19NA and *Serratia* N0-10LB strains in LB broth supplemented with ammonium sulphate, P in broth was significantly ($P \leq 0.05$) increased (4-5 μM P ml^{-1}) compared with the respective controls (2-3 μM P ml^{-1}). In contrast, with *Enterobacter* N0-29PA and *Pseudomonas* N1-5PA strains the P in broth was significant lower ($P \leq 0.05$) (3 μM P ml^{-1}) compared with controls (5 μM P ml^{-1}).

Table 3.2. Genetic characterization of selected efficient indole acetic acid producing phosphobacteria (IAAP).

Strains	Taxonomic	Closest relative or cloned sequences (Accession no.)[a]	Similarity (%)	Accession no.
Non-N-fertilised pasture (N0)				
N0-10LB	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Serratia grimesii* from soil (FJ469981)	97	JN050949
N0-20LB	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Serratia* sp. from soil (EU414474)	100	JN050950
N0-20NA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Enterobacter* sp. from soil (FN555403)	99	JN050951
N0-21NA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	Endophytic bacterium from rhizosphere (FJ603034)	96	JN050952
N0-22NA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	Uncultured bacterium from rhizosphere (GQ457013)	99	JN050953
N0-25PA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	Uncultured bacterium from rhizosphere (GQ457013)	100	JN050954
N0-29PA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Enterobacter* sp. from soil (FN555403)	100	JN050955
N0-6R2A	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Serratia proteamaculans* from wheat roots (EU627690)	99	JN050956
N0-20R2A	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Enterobacter* sp. from rhizosphere soil (GQ383912)	97	JN050957
N0-7NA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Enterobacter ludwigii* from from ryegrass rhizosphere (EU006530)	98	JN050958
N fertilised pasture (N1)				
N1-41LB	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Serratia* sp. from soil (FJ786070)	98	JN050959
N1-19NA	*Firmicutes; Bacilli; Bacillus*	*Bacillus thuringiensis* from soil (FJ655838)	73	JN050960
N1-29PA	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Serratia grimesii* from soil (FJ469981)	97	JN050961
N1-3R2A	*Bacteroidetes; Flavobacteria*	Uncultured bacterium from rice plant (AB114609)	81	JN050962
N1-55PA	*Proteobacteria; Gammaproteobacteria; Pseudomonas*	*Pseudomonas aeruginosa* from soil (FJ972538)	100	JN050963
N1-11aR2A	*Proteobacteria; Gammaproteobacteria; Enterobacteria*	*Enterobacter* sp. from soil (DQ985288)	99	JN050964
N1-11cR2A	*Firmicutes; Bacilli; Bacillus*	*Bacillus subtilis* from wheat field soil (FJ959367)	99	JN050965
N1-41PA	*Proteobacteria; Gammaproteobacteria; Pseudomonas*	*Pseudomonas aeruginosa* from soil (FJ972528)	99	JN050966
N1-52PA	*Proteobacteria; Gammaproteobacteria; Pseudomonas*	*Pseudomonas aeruginosa* from soil (FJ972530)	99	JN050967
N1-20R2A	*Bacteroidetes; Flavobacteria*	Bacterium from soil (AM932449)	72	JN050968

[a] Based on partial sequencing of 16S rRNA gene and comparison with Genbank database.

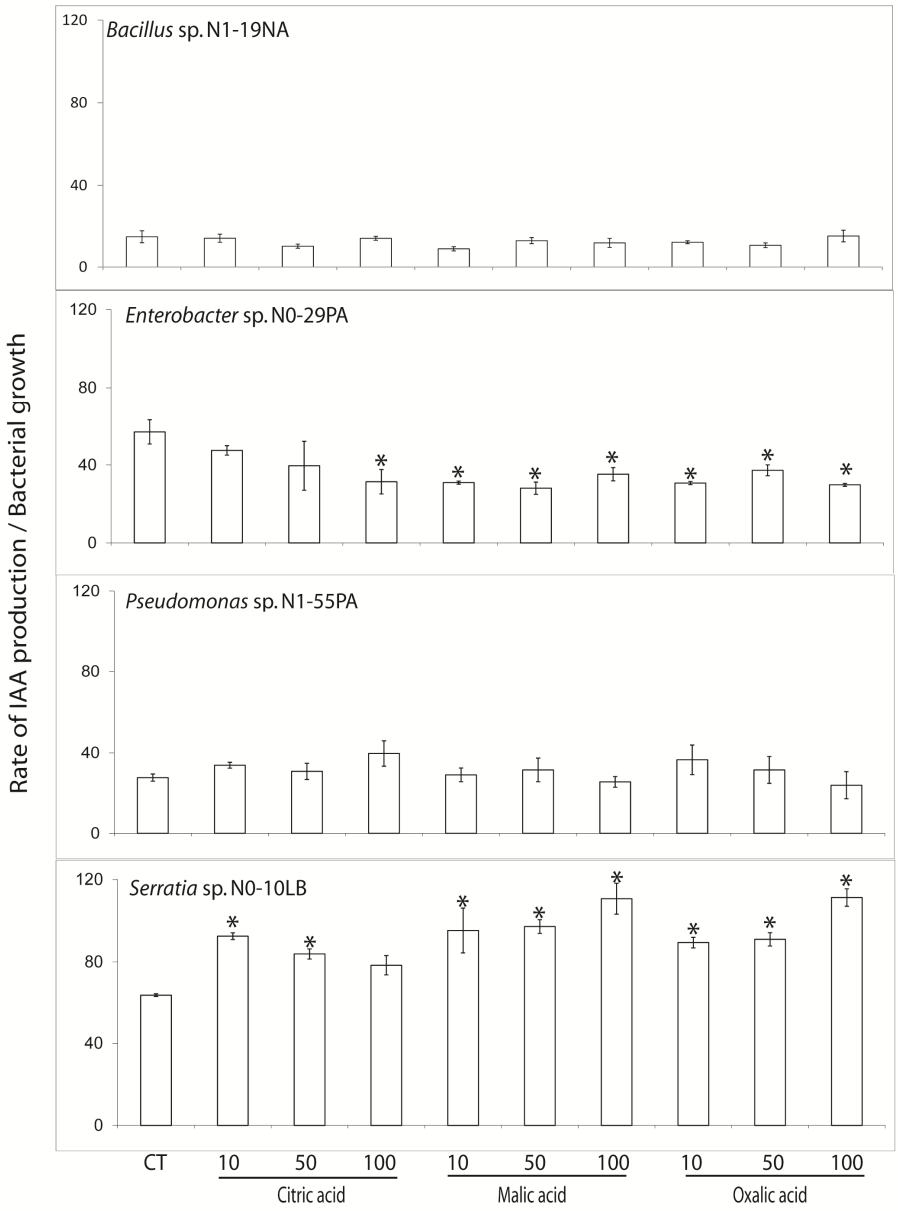

Figure 3.2. Effect of citric, malic and oxalic acids (10, 50 and 100 μM) on the IAA production by selected efficient indole acetic acid phosphobacteria (IAAP) in LB (pH 4.8) for 48 h at 30°C. Values represent mean±standard error (n=3).* denote significant difference ($P \leq 0.05$).

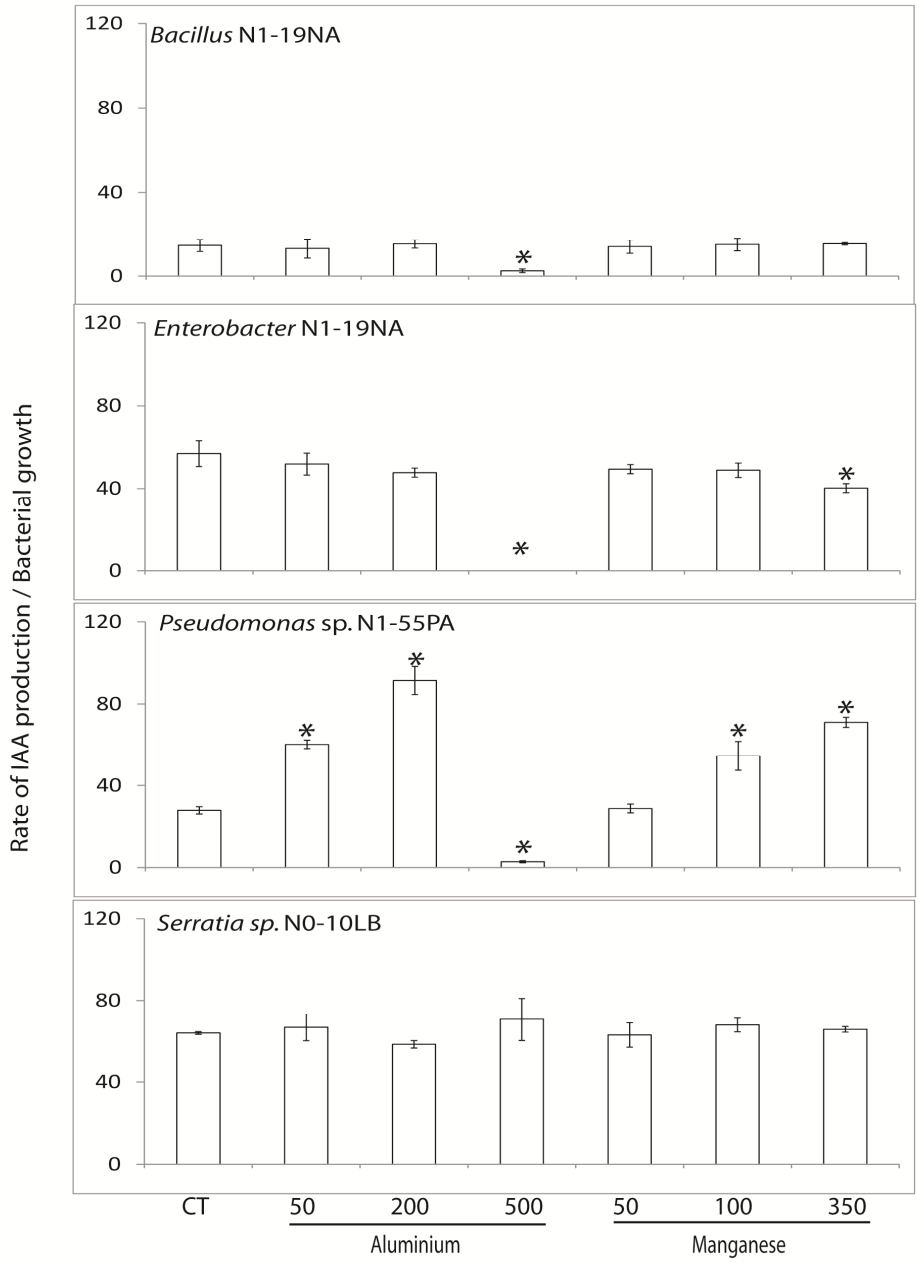

Figure 3.3. Effect of aluminium (50, 200 and 500 μM) and manganese (50, 100 and 350 μM) on the IAA production by selected efficient indole acetic acid phosphobacteria (IAAP) in LB (pH 4.8) for 48 h at 30°C. Values represent mean±standard error (n=3).* denote significant difference ($P \leq 0.05$).

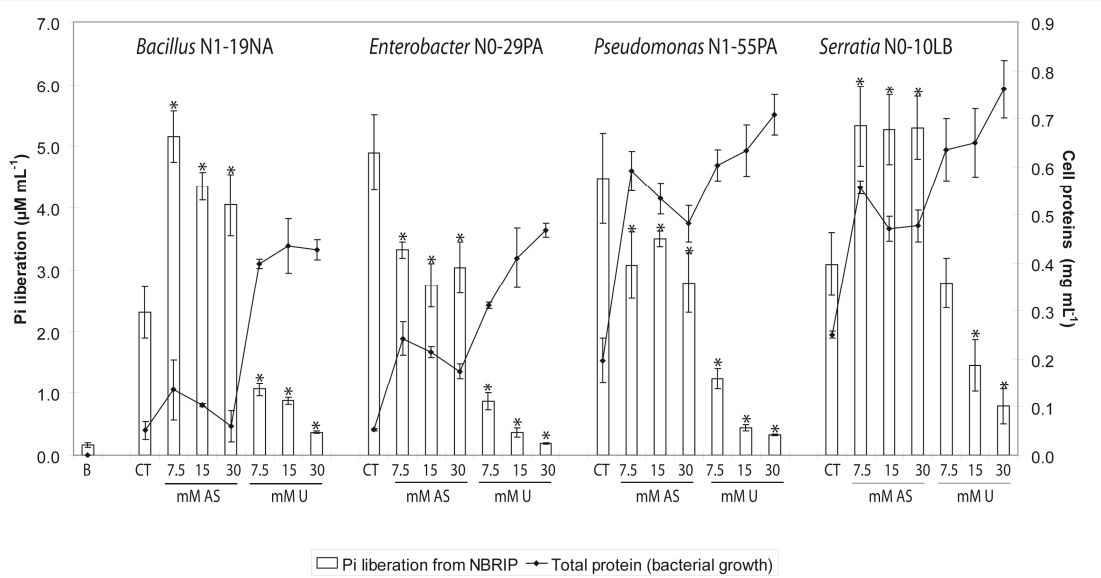

Figure 3.4. Effect of ammonium sulphate (AS) and urea (U) on Pi liberation by selected efficient indole acetic acid phosphobacteria (IAAP) in NBRIP broth (pH 7.0) for 48 h at 30°C. B: uninoculated blank; CT: inoculated control. Values represent mean±standard error (n=3).

3.4. **Discussion**

It is widely accepted that the diversity and/or functionality of microbial community of the rhizosphere can be influenced by a wide variety of environmental factors. Root exudates, pH, plant species, soil type, soil parental material and soil fertilisation may significantly alter the structure and diversity of the soil microbial community (Marschner et al. 2001; Fierer and Jackson 2006; Singh and Mukerji 2006; Ulrich and Becker 2006; Suzuki et al. 2009). In the present study, N fertilisation affected the natural occurrence of culturable rhizobacteria that have the ability to solubilise/mineralise insoluble P forms and produce IAA. However, it noteworthy that we studied culturable rhizobacteria which may represent only a small portion (1–10%) of total bacteria in the rhizosphere (Nannipieri et al. 2003).

This study also showed high percentages of phosphobacteria in Andisols, similar those reported by Jorquera et al. (2008, 2011). De Freitas et al. (1997) reported that 32% of isolates had the ability to mineralise Ca-phosphate. Likewise, Hussin et al. (2007) reported that approximately 83% of the bacterial isolates associated with maize roots produced extracellular phytases that could mineralise P from inositol hexaphosphate (phytic acid, dodecasodium salt). We also observed that long-term N fertilisation influenced the population densities of culturable phosphobacteria. There are only a few reports linking the occurrence of phosphobacteria with N fertilisation. One effect of N fertilisation is the alteration of nutrient dynamics and concentrations of soluble phosphate which are maintained by differences in relative demand for C, N, and P during organic matter decomposition (Harapiak et al. 2004). The N fertilisation also affects the composition and/or activity (enzymes) of microbial communities (Ge et al. 2010), although the specific effects on PGPR were not examined. Poonguzhali et al. (2006) reported that high N

fertilisation (320 kg N ha^{-1}) resulted in selection for higher percentages (63%) of PS bacteria from an acidic soil. This higher proportion of culturable phosphobacteria in N fertilised acidic soils should be related to the release of P by rhizobacteria as a tolerance mechanism against Al^{3+} toxicity because Al^{3+} forms complexes with phosphates in acidic soils (Borie et al. 1989). Likewise, the N-fixing cyanobacterium, *Anaboena cylindrical*, can accumulate intracellular inorganic polyphosphate granules used to sequester Al^{3+}, and this mechanism may be responsible for detoxifying limited amounts of Al^{3+} (Pettersson et al. 1985). However, it is necessary to mention that the solubilisation/mineralisation of P is a complex phenomenon in the nature and *in vitro* studies with culturable phosphobacteria may not be correlated with the major available of P in the rhizosphere and the promotion of plant growth (Chung et al. 2005; Collavino et al. 2010).

Here a total of 1,176 rhizobacterial strains were examined, from which 28% of the strains were characterised as culturable IAA-producing rhizobacteria, independently of their ability to solubilise/mineralise P. Several studies have reported IAA production by diverse isolates of rhizobacteria. Khalid et al. (2004a; 2004b) reported that approximately 70-80% of the bacteria isolated from cereal rhizospheres were capable of producing IAA in the absence of L-tryptophan and that 100% of the bacteria were capable of producing IAA in the presence of L-tryptophan. Naik et al. (2008) observed that 49% of the PS fluorescent pseudomonad strains isolated from rice and banana rhizospheres are able to produce IAA. In contrast, other studies have revealed lower percentages of IAA-producing bacteria occurrence. Ramos et al. (2006) observed that 17.6% of rhizobacteria isolated from *Gum cistus* rhizospheres are IAA-producing bacteria. Hynes et al. (2008) reported that only 7% of bacteria isolated from pea, lentil and chickpea rhizospheres are capable of producing IAA *in vitro*. The differences in the percentages of culturable IAA-producing rhizobacteria

occurrences may to be related to differences in isolation methods, number of screened isolates, the types of plants and soils, and growth conditions.

Coincident with our results, Yuan et al. (2011) studied the influence of chemical and organic N fertilisation (0 and 400 kg N ha^{-1} yr^{-1}) on IAA-producing rhizobacteria. They reported that the highest diversity of IAA-producing bacteria was observed in unfertilised soils or in soils amended with organic N fertilisers, whereas application of high levels of chemical N fertilisers reduced the numbers of species that produce IAA. These authors concluded that different fertilisation treatments affect the IAA yield mainly through modifying the occurrence of rhizobacteria rather than IAA-production activity. However, the effect of N fertilisation on the occurrence of IAA-producing rhizobacteria is not yet well understood.

The present study also revealed that approximately 66% of isolates showed more than one mechanism and 10-13% of the isolates contain simultaneously the three of the mechanisms analysed (IAAP). Identification of the 20 efficient IAAP strains by partial sequencing of the 16S rRNA genes revealed similarities with known bacteria belonging to genera *Bacillus, Enterobacter, Flavobacteria, Pseudomonas* and *Serratia* (Table 3.2). This result is in agreement with previous PGPR studies that have described the isolation of *Bacillus* (Ahmad et al. 2008), *Enterobacter* (Collavino et al. 2010) and *Pseudomonas* (Ahmad et al. 2008; Naik et al. 2008). From the rhizospheres of perennial ryegrass pastures grown in volcanic acidic soils, Shoebitz et al. (2009) isolated and identified an *Enterobacter ludwigii* strain with multiple traits, such as nitrogenase activity, mineral phosphate solubilisation activity, antifungal activity and IAA-producing ability. It has been postulated that many PGPR strains act by more than one mechanism involved in plant growth promotion, and that the most effective strains of PGPR may express several

mechanisms acting simultaneously and/or sequentially (Ahmad et al. 2008). However, the exact mechanisms by which PGPR with multiple traits promote plant growth are not fully understood. Still other factors are the relative expression levels of these traits over time, and whether the PGPR are located in effective population densities at the microsites on plant roots where root growth can be modulated by IAA production, ethylene suppression, hydrogen cyanide, and availability of nutrients.

In relation to factors evaluated, several studies have suggested that the effectiveness of PGPR may be mediated by various environmental factors (Ahmad et al. 2008; Park et al. 2010; Yuan et al. 2011). However, only a few studies have examined the effects factors that are relevant in volcanic soils on effectiveness of PGPR. Our results showed that the presence of organic acids may stimulate (*Serratia* sp. N0-10LB) or inhibit (*Enterobacter* sp. N0-29PA) the IAA production by rhizobacteria. It is known that organic acids affect diverse important processes in the rhizosphere (Jones1998), such as solubilising of nutrients and metals chelating, but to our knowledge there are no reports on the effect of organic acids on PGPR traits. With respect to metals, the presence of Al and Mn stimulated the IAA production by *Pseudomonas* sp. N1-55PA. To our knowledge there is not report indicating the stimulation of IAA production linked to increase of Al and Mn. We hypothesised that this effect might be related to a defence strategy of this strain. It is noteworthy that *Serratia* sp. N0-10LB were not affected by highest Al concentration (500 μM) respect to other strains and control without Al addition. This result indicate that *Serratia* sp. N0-10LB is well adapted to Al stress condition without lose its ability to produce IAA.

According to the P-release assay, the content of inorganic P in broth was affected by the type of N (ammonium sulphate and urea) source and type of rhizobacteria. Among the

four strains examined, all showed a higher growth when liquid medium was supplemented with urea than with ammonium sulphate. This higher bacterial growth indicates a higher solubilising of supplemented P, with the consequent P immobilization in cell biomass and low P content in broth. Park et al. (2010) reported that urea is the better N source as compared with ammonium sulphate for P solubilisation by the *Burkholderia vietnamiensis* M6 strain. However, these authors indicated that urea concentrations above or below 0.015% (w/v) result in decreased levels of P solubilisation. In contrast, Sharan et al. (2008) observed higher P solubilisation carried out by *Xanthomonas campestris* in the presence of ammonium sulphate than with urea.

Our study suggests that each factor we examined could both positively or detrimentally affect the population size and activity of individual strains, which confounds the overall goal of deriving general relationships for the effects on environmental factors on specific PGPR functions. However, it may still be possible to derive general principles using quantitative methods to measure net changes in copy numbers and expression of these functions in relation to changes in specific environmental factors. Also, the characterization of individual strain responses can have application in identifying strains that may be used as soil inoculants in situations where PGPR activities might be enhanced by inoculation. For example, the *Serratia* sp. N0-10LB strain was not affected by metals. Thus, this bacterial strain may be better adapted to changing conditions in the rhizosphere under acidic conditions, such Chilean Andisols.

3.5. **Conclusions**

In the present work, we show that the N fertilisation (as urea) in pastures established in Chilean Andisols plays a key role in the occurrence of potential plant growth-promoting rhizobacteria (PGPR). Thus, the long-term N fertilisation significant increases (30%) the occurrence of culturable rhizobacteria that solubilise/mineralise insoluble phosphorus forms and decrease (7%) the occurrence of culturable rhizobacteria with ability to produce indole acetic acid. Moreover, the percentage of potential PGPR was higher in N-fertilised pasture (88%) compared with non-N-fertilised (69%). The most efficient indole acetic acid phosphobacteria (IAAP) were identified as *Enterobacter*, *Serratia* sp., *Pseudomonas* sp. and *Bacillus* sp. It is noteworthy that efficient IAAP belonging to *Pseudomona*s and *Bacillus* genus were only found in N fertilized pastures. However, it is necessary indicate that this study is based on culturable bacteria which can represent only 1-10% of total bacterial populations. The *in vitro* assays about the effect of organic acids and toxic metals and on IAA production by efficient IAAP showed that the ability to produce IAA can be increased 2-5 fold by metals presence in *Pseudomonas* sp. N1-55PA and 31-74% by organic acids in *Serratia* sp. N0-10LB. In relation to N supply, our observations at 48 h showed that the addition of urea resulted in a higher bacterial growth, compared with ammonium sulphate, indicating a quick P immobilization in cell biomass, but with the consequent decrease in P in media.

Summarizing, in this study we demonstrate that N fertilisation and factors present in Chilean Andisols have a relevant role in the occurrence and performance of culturable rhizobacteria containing beneficial traits for plant growth. Furthermore, this influence

should be taken into account when microbial inoculants are developed for applying them in volcanic acidic soils.

3.6. **References**

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Chapter 4

Effect of Rhizobacteria Inoculation on Plant and Rhizosphere Parameters of Cereals Grown Under P-deficiency

(*Manuscript in preparation*)

Abstract

Cereal production in southern Chile is based on volcanic soils (Andisol) with low availability of phosphorus (P). Releasing of bound P in soil by bacterial inoculants is an important tool to be considered to improve growth and efficiency of crops. However, what is the potential contribution of rhizobacteria on cereals grown under P-deficient Andisols remain unknown. Pot experiments in a greenhouse were conducted to investigate the contribution of four selected native rhizobacteria on cereal plants grown in an Andisol with basal and without P-fertilization. Wheat, oat and barley plants were grown in Piedras Negras Andisol Serie (3 mg kg^{-1} P_{Olsen}) and inoculated with selected native rhizobacteria (*Bacillus* sp. N1-19NA, *Enterobacter* sp. N0-29PA, *Pseudomonas* sp.N1-55PA and *Serratia* sp. N0-10LB). Plant dry biomass, plant P uptake and P concentration, rhizosphere soil enzymatic activities, root superoxide dismutase (SOD) activity and changes in rhizosphere bacterial communities were evaluated. *Enterobacter* N0-29PA significantly increased the P concentration in wheat plant grown in sterile soil, and plant dry biomass and P uptake of oat plants grown in non-sterile soil. In general, the inoculation with the other rhizobacteria (*Bacillus* sp. N1-19NA, *Pseudomonas* sp.N1-55PA and *Serratia* sp. N0-10LB) did not affect the plant biomass, P uptake and P concentration in cereal plants.. The DGGE technique did not also reveal consistent differences between rhizosphere bacterial communities among treatments in each of the cereal species. Although,, the inoculation with these rhizobacteria produced changes on acid phosphatase and urease activities in the rhizosphere and increased the potential of IAA production in the rhizosphere. Interesting, the inoculation with *Enterobacter* N0-29PA, *Pseudomonas* N1-55PA and *Serratia* N0-10LB significantly increased the antioxidant root enzyme SOD in wheat plants. These results

shows that under P-deficient conditions, the rhizobacteria inoculation not only can influence the plant growth, P uptake and P concentration of cereal plants but also can affect other relevant as enzymatic rhizosphere activity and plant defense mechanisms agaisnt stress.

Keywords: Andisol, indole acetic acid, phosphate, phytate, rhizobacteria.

4.1. **Introduction**

Cereal production in southern Chile is based on ash-derived volcanic soils (Andisols). Cultivated Andisols present high content of total phosphorus (P), but the availability of P is low to plants (Borie and Rubio 2003; Turner 2007). The low P availability is due that Chilean Andisols have a high P fixing capacity and the P content in soil is increased by added fertilizer P by farmers which is quickly adsorbed forming complexes with minerals, allophane, and ferrihydrite (Mora and Canales 1995a, 1995b), thus large amounts of P fertilizers are needed to overcome P limitations (Mora et al. 1999). Under this scenario, the importance of plant growth-promoting rhizobacteria (PGPR) populations on mineral plant nutrition, particularly P, is well recognized (Bowen and Rovira 1999; Martínez-Viveros et al. 2010). Improving fertility by releasing bound P in soil by PGPR is an important aspect to be considered for achieving optimum crop growth. In this context, phosphate solubilization, phytate mineralization and indole acetic acid (IAA) production are beneficial mechanisms desired in PGPR.

Phosphate-solubilizing rhizobacteria are common components of soils, and they are well known to enhance the plant growth (Rodríguez and Fraga 1999; Yu et al. 2011). On the other hands, IAA-producing rhizobacteria have also been reported to significantly enhance the development of the host plant root system, which indirectly may improve P uptake by plants (Marschner et al. 2011). Despite that organic P, particularly phytate, representing an important source of P potentially available to plant in Andisols, scarce studies have been conducted to evaluate the role of rhizobacteria to release available P from phytate in soils. It has been postulated that phytate-mineralizing rhizobacteria could play a predominant role in the recycling of phytate from soil P reserves (Richardson 2001). Recent studies have

isolated phosphobacteria (phytate-mineralizing and phosphate-solubilizing bacteria) from the rhizosphere the plants grown in Chilean Andisols and they have been suggested as potential soil inoculants to increase P availability (Jorquera et al. 2008; Shoebitz et al. 2009; Jorquera et al. 2011). These native isolates have also included rhizobacteria containing other mechanisms found in PGPR, such as IAA production and siderophore (Shoebitz et al. 2009; Acuña et al. 2011). Moreover, commom agricultural practices (such as fertilization with urea) and environmental factors (pH, organic acids and cation metals) have shown to be relevant in the occurrence and performance of potential PGPR in Chilean Andisols (Martínez et al. 2011).

However, what is the real contribution of native rhizobacteria on plant growth and P concentration in cereals grown in Chilean Andisols remains unknown. Thus, the objectives of this research were to evaluate the inoculation of native rhizobacteria on plant (total biomass, P uptake, P concentration and activity of root-surface phosphatase, superoxide dismutase and thiobarbituric acid-reactive-substance) and rhizospheric (P available, IAA potential, activity of urease and phosphatase and structure of bacterial community) parameters of cereal plants grown in an Andisol with low P available.

4.2. **Materials and methods**

Rhizobacteria

The strains *Bacillus* sp. N1-19NA, *Enterobacter* sp.N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB were previously isolated from pasture rhizosphere and selected for their ability to solubilize phosphate, mineralize phytate and produce indole acetic acid (IAA) under laboratory conditions (Martínez et al. 2011).

Soil

The soil chosen is one Andisol belonging Piedras Negras soil (PNS) Serie. The soil samples were collected from the top 20 cm in pasture located in Osorno province from southern of Chile (40º20'S; 72º35'W). Soil collected were air-dried and sieved to 2 mm. The main chemical soil properties are (mg kg^{-1}): total P (P_t) 1,124, inorganic P (Pi) 131 and organic P (Po) 993, P_{Olsen} 3, K 109, pH_{H20} 5.4, organic matter 17 (%) and Al saturation 15.2 (%), cation exchange capacity 2.95 (cmol+ kg^{-1}).

Greenhouse experiment I

A pot experiment was conducted to evaluate the effect of rhizobacteria inoculation on P content by wheat (*Triticum aestivum* L. cv. Fritz) plants. For this experiment sterile and non-sterile soil was utilized. Soil at 70% moisture was sterilized in 1 kg plastic bags using a microwave (5 min to 2,450 MHz) for three consecutive days (Borie and Rubio 1999), and

then aerated for 24 hours.The wheat seeds were inoculated with rhizobacteria (~ 1×10^8 cfu $seed^{-1}$). The seeds were coated with the bacterial pellet before sown. After germination, 27 plants per pot were sowed and plantlets were re-inoculated with a bacterial suspension (10^9 CFU ml^{-1} $plant^{-1}$) at 10 days after germination (Fernández et al. 2007). Soil basal P fertilization (100 mg kg^{-1}) was applied as triple superphosphate before sown. Wheat plants were irrigated regularly to maintain 60% of the maximum water holding capacity. Nitrogen fertilization (50 mg kg^{-1} as urea) was carried out at 9 day.

A completely random experimental design was adopted. The inoculation treatments included: (1) control (non-inoculated), (2) *Bacillus* sp. N1-19NA, (3) *Enterobacter* sp.N0-29PA, (4) *Pseudomonas* sp. N1-55PA, (5) *Serratia* sp. N0-10LB and (6) mix of four strains. These treatments were applied to both sterile and non-sterile soil.

The wheat plants were maintained under greenhouse conditions for 30 days. Wheat plants were harvested and analyzed for plant biomass and P concentration in tissues (see below method description).

Greenhouse experiment II

A pot experiment was conducted with wheat, oat (*Avena sativa* L. cv. Rayen) and barley (*Hordeum vulgare* L. cv. Pincoya) plants. Cereal plants were germinated on wet filter paper for 7 days and then the seedlings were transplanted to pots (10 plants per pot) filled with 0.95 kg of PNS, and irrigated regularly to maintain 60% of the maximum water holding capacity. The cereal plants were inoculated with a bacterial suspension (10^9 CFU mL^{-1} $plant^{-1}$) at 1, 30 and 55 days (Fernández et al. 2007). A completely random experimental design was adopted. The inoculation treatments included: (1) control (non-inoculated), (2)

Bacillus sp. N1-19NA, (3) *Enterobacter* sp.N0-29PA, (4) *Pseudomonas* sp. N1-55PA, and (5) *Serratia* sp. N0-10LB. These treatments were applied to wheat, oat and barley. The plants were maintained under greenhouse conditions for 75 days and N fertilization (equivalent to 200 kg urea ha^{-1}) was carried out at 30 day. The basal P fertilization was not applied.

In the end of this experiment, cereal plants were harvested and the following variables were measured: plant biomass, P concetration in tissues, P available in the rhizosphere, root surface phosphatase activity, soil enzymes (acid phosphatese and urease), IAA potential in the rhizosphere, root superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBAR) and bacterial community composition in rhizosphere.

Biomass and P concentration of plants

Plants were carefully removed from the pots and then washed with distilled water for removing soil trapped in the roots. Shoots and roots were separated, rinsed with deionized water and dried at 65ºC for 48 h to determine dry weight (dw). The shoot and root P concentration in tissues was analyzed by the molybdo-vanadate method as described by Sadzawka et al. (2007). Briefly, after dry weight determination, samples were dry-ashed in a muffle furnace at 500°C for 8 h and digested with 2 M HCl. Ashes were filtered and P in the extracts was quantified in a spectrophotometer at 466 nm.

P availability and root surface phosphatase activity

Soil available P in the rhizoshere was estimated by using the molybdenum blue method as described by Sadzawka et al. (2006). Briefly, available P is extracted from rhizospheric soil by bicarbonate ($NaHCO_3$) solution at pH 8.50. P in the extracts was quantitatively analyzed at 880 nm by a formation of an antimony–phosphate–molybdate complex reduced with ascorbic acid to form a blue-colored complex.

In relation to root surface phosphatase activity, the roots were carefully removed from soil, and root surface phosphatase activity was determined using *p*-nitrophenylphosphate (*p*-NPP) substrate according to the procedure described by Rubio et al. (1990). Roots were submerged in *p*-NPP solution and incubated for 30 min at 20 º C, with roots in the dark and leaves were uncovered. Acid phosphatase activity was calculated as the gram of *p*-nitrophenyl (*p*-NP) released during 1 min of assay per gram of root fresh weight.

Soil enzymes and IAA potential

Acid phosphatase and urease were chosen as indicators of soil capacity to mineralize organic N and P compounds. Soil acid phosphatase activity was measured using the method described by Tabatabai and Bremner (1969) and modified by Rubio et al. (1990) for soil with high organic matter content. Samples of 1 g in 4 mL buffer (0.1 M Tris, pH 5.5) were incubated with 0.115 M *p*-NPP for 1 h at 20 ºC in water bath. The reaction was stopped by the addition of 1 mL of 0.5 M $CaCL_2$, and the mixture was filtered and centrifuged at 2,500

g for 10 min. The *p*-NP concentration in the supernatant was measured at 400 nm in a spectrophotometer. Urease activity was assayed by the modified method of Nannipieri et al. (1980). Four mL of 0.1 M phosphate buffer (pH 7.1) and 1 mL of 1.067 M urea were added to 1 g soil samples. The samples were incubated at 20 ºC for 2 h, and thereafter, 5 mL of 2 M KCl were added to terminate the reaction. The $N\text{-}NH_4^+$ was determined by ion selective electrode.

To determine the potential of indole acetic acid (IAA) activity in rhizosphere the method described by Benitez et al. (2004) was used. Two grams of fresh rhizosphere soil was placed in a 50 ml flask. Six ml of phosphate buffer (pH 7.5) with glucose (1 g glucose in 100 ml^{-1} phosphate buffer) and 4 ml of 4 mM L-tryptophan were added. Soil suspensions were mixed and incubated at 37ºC for 24 h in the dark. Two milliliters of 5% trichloroacetic acid solution (to inactivate enzymes), and 1 ml of 0.5 M $CaCl_2$ solution were added. An aliquot was transferred to a 1.5 mL microtube and centrifuged at 5,000 g for 5 min. The IAA concentration was measured by spectrophotometryat 535 nm.

Root superoxide dismutase and thiobarbituric acid reactive substances

The enzyme antioxidants superoxide dismutase (SOD) is the first line of defense against reactive oxygen species (ROS) and commonly used as indicator of plant response to stress. Root samples were frozen in liquid nitrogen and stored at −70 ◦C. Later, the frozen material was homogenized and centrifuged. SOD activity was assayed by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The absorbance was measured at 560 nm. One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of the NBT reduction in comparison to tubes lacking enzymes at 25◦C

(Donahue et al. 1997). The results were corrected by quantification of total protein content (Bradford) in root biomass (Kruger 2002). In parallel, thiobarbituric acid-reactive-substances (TBARS) assay was used to estimate peroxidation of lipids in membrane as indicator of plant root stress. In the fresh material, lipid peroxidation was assayed by measuring the TBARS according to method described by Ederli et al. (1997) and Hodges et al. (1999). Fresh control and treated roots (0.15 g) were homogenized with trichloroacetic acid and thiobarbituric acid. The amount of TBARS was determined in the soluble fraction by measuring their absorbance at 532, 600 and 440 nm in order to correct the interference generated by TBARS–sugar complexes.

Bacterial community composition in the rhizosphere

The analysis of bacterial community composition in rhizosphere soil was evaluated by denaturing gradient gel electrophoresis (DGGE) technique based on 16S rRNA gene. The DGGE procedure was carried out as described by Jorquera et al. (2010). Extraction of total DNA from soil samples was carried out by using PowerSoil Soil DNA Isolation Kit (Mo–Bio Laboratories, Inc.) and fragments of 16S rRNA gene were amplified by PCR with the primer set EUBf933-GC/EUBr1387 (Iwamoto et al. 2000). The DGGE analysis was performed in a 9% (w/v) polpolyacrylamide gel with a gradient of 30% and 55% (urea and formamide).The electrophoresis was run for 12 h at 100 V and gel stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on an UV transilluminator. Clustering of DGGE banding profiles using a dendrogram was also carried out by using Phoretix 1D analysis software (TotalLab Ltd.).

Statistical Analysis

The data were analysed by a one-way analysis of variance (ANOVA), and comparisons were carried out for each pair with Student's t-test using JMP statistical software (SAS Institute, Inc.). All experiments were carried out in triplicate, and the values are given as means ± standard errors. Differences were considered to be significant when the *P* value was less than or equal to 0.05.

4.3. **Results**

Greenhouse experiment I

The effect of rhizobacteria on dry biomass, P uptake and P concentration of wheat plants grown in sterile and non-sterile soil is shown in Table 4.1. Compared with uninoculated control, the result showed that none of inoculated rhizobacteria significantly ($P \leq 0.05$) increased plant biomass in sterile soil (Table 4.1). Similarly, the inoculation of wheat with selected rhizobacteria did not significantly ($P \leq 0.05$) increased the P uptake compared with controls (Table 4.1). In contrast, P concentration in plants grown in sterile soil and inoculated with *Enterobacter* sp. N0-29PA and *Bacillus* N1-19NA was significantly ($P \leq 0.05$) increased (6.5% and 7.3%, respectively) compared with uninoculated controls. In non-sterile soil, the inoculation with *Enterobacter* sp. N0-29PA also increased (7.6%) the P concentration in inoculated plants as compared with control.

Greenhouse experiment II

Growth and P content of plants

In general terms, the inoculation of wheat and barley with *Bacillus* sp. N1-19NA, *Enterobacter* sp. N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB did not significantly ($P \leq 0.05$) increased the plant biomass compared with controls (Table 4.2). Except in oat where the inoculation with *Enterobacter* sp. N0-29PA significantly ($P \leq 0.05$) increased (29%) the plant biomass compared with control.

Similarly, wheat and barley plants inoculated with selected rhizobacteria did not significantly ($P \leq 0.05$) increased the total P uptake compared with controls (Table 4.2). Hovewer, oat inoculated with *Enterobacter* sp. N0-29PA significantly ($P \leq 0.05$) increased (47%) total P uptake compared with control.

In relation to P concentration, the rhizobacterial inoculation did not significantly ($P \leq 0.05$) increase the P concentration in plants compared with control (Table 4.2). However, the higher P concentrations (7-14%) were observed in plants inoculated with *Enterobacter* sp. N0-29PA (Table 4.2).

Table 4.1. Effect of rhizobacteria inoculation on wheat biomass, P uptake and P concentration in sterile and non-sterile volcanic soil with basal P fertilization.

Sterile soil	Plant dry biomass (g pot^{-1})	P uptake (mg)	P concentration (mg kg^{-1})
Control non-inoculated	2.48 ±0.06 a	3.77 ±0.12 ab	1522 ±10 b
Bacillus N1-19NA	2.46 ±0.02 a	4.00 ±0.05 a	1621 ±11 a
Enterobacter N0-29PA	2.18 ±0.02 b	3.55 ±0.03 bc	1633 ±8 a
Pseudomonas N1-55PA	2.21 ±0.06 b	3.36 ±0.08 c	1517 ±8 b
Serratia N0-10LB	2.25 ±0.08 b	3.50 ±0.05 c	1558 ±36 b
Mix	2.30 ±0.01 ab	3.56 ±0.03 bc	1547 ±3 b
Non-sterile soil	Plant dry biomass (g pot^{-1})	P uptake (mg)	P concentration (mg kg^{-1})
Control non-inoculated	1.80 ±0.08 a	2.79 ±0.12 a	1557 ±74 ab
Bacillus N1-19NA	1.99 ±0.05 a	3.09 ±0.04 a	1550 ±19 ab
Enterobacter N0-29PA	1.75 ±0.06 a	2.93 ±0.06 a	1676 ±27 a
Pseudomonas N1-55PA	1.82 ±0.13 a	2.90 ±0.23 a	1590 ±14 ab
Serratia N0-10LB	1.81 ±0.01 a	2.68 ±0.06 a	1481 ±24 b
Mix	1.89 ±0.08 a	3.12 ±0.11 a	1650 ±14 a

Values represent mean ± standard error (average of three repeats). Different letters in the same column denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Student's t-test using JMP).

Table 4.2. Effect of rhizobacteria inoculation on plant biomass, P uptake and P concentration in wheat, oat and barley grown in non-sterile soil and without P-fertilization.

Wheat	Plant dry biomass (g pot^{-1})	P uptake (mg)	P concentration (mg kg^{-1})
Control non-inoculated	2.90 ±0.06 a	2.45 ±0.11 a	845 ±28 ab
Bacillus N1-19NA	2.33 ±0.17 b	1.83 ±0.11 b	787 ±12 b
Enterobacter N0-29PA	2.76 ±0.24 ab	2.39 ±0.25 ab	863 ±16 ab
Pseudomonas N1-55PA	2.50 ±0.04 ab	2.31 ±0.10 ab	921 ±31 a
Serratia N0-10LB	2.75 ±0.04 ab	2.43 ±0.11 a	883 ±36 ab
Oat	Plant dry biomass (g pot^{-1})	P uptake (mg)	P concentration (mg kg^{-1})
Control non-inoculated	2.49 ±0.12 b	1.77 ±0.14 b	713 ±43 ab
Bacillus N1-19NA	2.82 ±0.16 ab	2.15 ±0.20 ab	759 ±31 ab
Enterobacter N0-29PA	3.21 ±0.02 a	2.61 ±0.15 a	812 ±44 a
Pseudomonas N1-55PA	2.73 ±0.10 b	1.83 ±0.17 b	667 ±41 b
Serratia N0-10LB	2.74 ±0.12 b	2.05 ±0.15 ab	745 ±21 ab
Barley	Plant dry biomass (g pot^{-1})	P uptake (mg)	P concentration (mg kg^{-1})
Control non-inoculated	1.37 ±0.09 a	0.89 ±0.04 a	656 ±16 a
Bacillus N1-19NA	1.24 ±0.10 a	0.82 ±0.08 a	659 ±22 a
Enterobacter N0-29PA	1.30 ±0.15 a	0.92 ±0.11 a	703 ±7 a
Pseudomonas N1-55PA	1.48 ±0.10 a	1.03 ±0.06 a	698 ±14 a
Serratia N0-10LB	1.20 ±0.01 a	0.81 ±0.02 a	674 ±17 a

Values represent mean ± standard error (average of three repeats). Different letters in the same column denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Student's t-test using JMP).

Phosphorus availability and root surface phosphatase activity

A significant ($P \leq 0.05$) higher P availability in the rhizosphere was only observed in wheat and oat plants inoculated with *Enterobacter* sp. N0-29PA compared to the uninoculated control, but the phosphatase activity of root surface was decreased the (Fig. 4.1, A and B). In oat and barley plants, the inoculation with *Bacillus* sp. N1-19NA also significantly ($P \leq 0.05$) increased soil available P and decreased the activity of root surface phosphatase activity compared to the uninoculated control (Fig. 4.1, B and C).

Soil enzyme and IAA potential

The application of rhizobacterial strains produced changes on acid phosphatase and urease activities in the rhizosphere (Table 4.3). In wheat, the acid phosphatase activity was significantly decreased (27%) by *Pseudomonas* sp. N1-55PA inoculation. In contrast, the application of the *Pseudomonas* sp. N1-55PA significantly ($P \leq 0.05$) increased the acid phosphatase (12%) and urease (32%) activities in oat compared with uninoculated control. On the other hands, the application of the *Enterobacter* sp. N0-29PA showed a significant ($P \leq 0.05$) increase (16%) of acid phosphatase and *Serratia* sp. N0-10LB showed a significant decreased (25%) of urease activity in barley plants compared with control.

In relation to IAA potential in the rhizosphere, in general term the inoculation with rhizobacteria increase the IAA production potential in the rhizosphere soil of plants (Fig. 4.2). The Figure 4.2A shows that the rhizobacteria inoculation significantly ($P \leq 0.05$) increased (73-125%) the potential of IAA in the rhizosphere of wheat soil. In oat, the treatments with rhizobacteria had a higher (28-52%) IAA production but not

significant ($P \leq 0.05$) compared with uninoculated control (Fig. 4.2B). Similarly to wheat plants, the inoculation with *Enterobacter* sp. N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB significantly ($P \leq 0.05$) increased (24-69%) IAA production in the rhizosphere of barley plants (Fig. 4.2C).

Root superoxide dismutase and thiobarbituric acid reactive substances

In relation to parameters involved in stress tolerance mechanisms of plants, the inoculation with rhizobacteria (except *Bacillus* sp. N1-19NA) significantly ($P \leq 0.05$) increased the antioxidant root enzyme SOD of wheat plants compared with uninoculated control (Fig. 4.3A) at the same level of stress indicated by TBARS. This respond was not observed with the rhizobacteria inoculation of oat and barley, where SOD did not enhance respect to the control (Fig. 4.3, B and C).

Table 4.3. Effect of rhizobacterial strains inoculation on rhizospheric soil enzyme activities in wheat, oat and barley crops.

Parameter	Treatment				
	Control	*Bacillus* N1-19NA	*Enterobacter* N0-29PA	*Pseudomonas* N1-55PA	*Serratia* N0-10LB
Wheat					
Acid phosphatase (mg *p*-NP g^{-1} soil h^{-1})	614 ±40 ab	683 ±17 a	649 ±18 ab	446 ±26 c	569 ±5 b
Urease (µmol NH_3 g^{-1}soil h^{-1})	4.05 ±0.3 ab	3.38 ±0.03 b	4.14 ±0.17 a	3.64 ±0.01 ab	3.81 ±0.04ab
Oat					
Acid phosphatase (mg *p*-NP g^{-1} soil h^{-1})	789 ±12 b	824 ±18 ab	836 ±0 ab	887 ±27 a	832 ±10 ab
Urease (µmol NH_3 g^{-1}soil h^{-1})	5.04 ±0.33 b	5.02 ±0.4 b	6.19 ±0.37 ab	6.65 ±0.63 a	6.57 ±0.16 a
Barley					
Acid phosphatase (mg *p*-NP g^{-1} soil h^{-1})	605 ±6 b	601 ±15 b	703 ±29 a	665 ±6 ab	632 ±9 ab
Urease (µmol NH_3 g^{-1}soil h^{-1})	8.9 ±0.8 a	8.6 ±0.09 ab	8.5 ±0.55 ab	9.2 ±0.24 a	6.7 ±0.16 b

Values represent mean ± standard error (average of three repeats). Different letters in the same row denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Student's t-test using JMP).

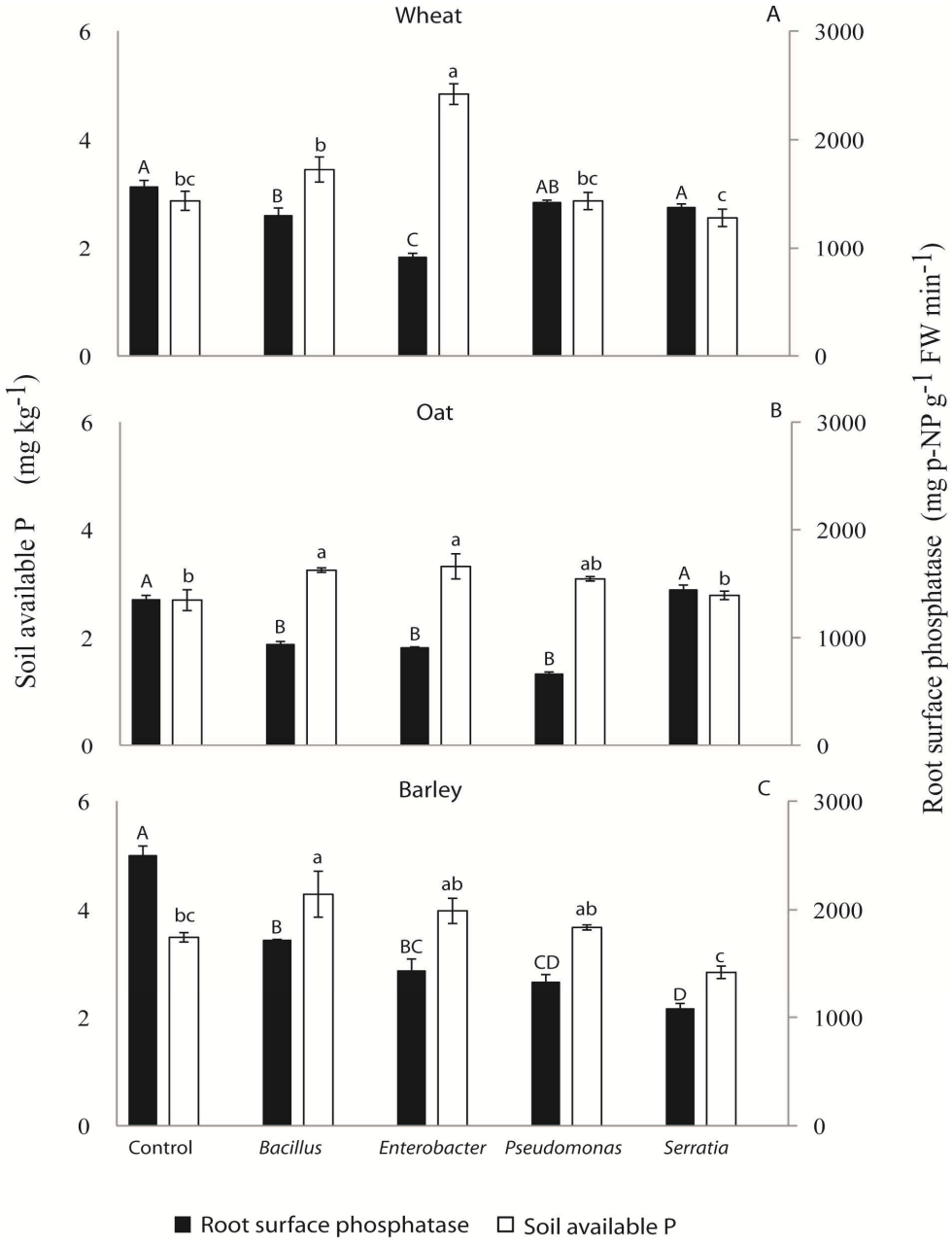

Figure 4.1. Available phosphorus in the rhizosphere soil and root surface phosphatase activity from inoculated plants of A) wheat, B) oat and C) barley. For each parameter, means (± standard error). Different uppercase letters indicate differences (P<0.05, comparisons of means were carried out for each pair with Student's t-test using JMP) between treatments respect to root surface phosphatase activity. Different lowercase letters indicate statistically significant differences (P<0.05, comparisons of means were carried out for each pair with Student's t-test using JMP) between treatments respect to soil available P.

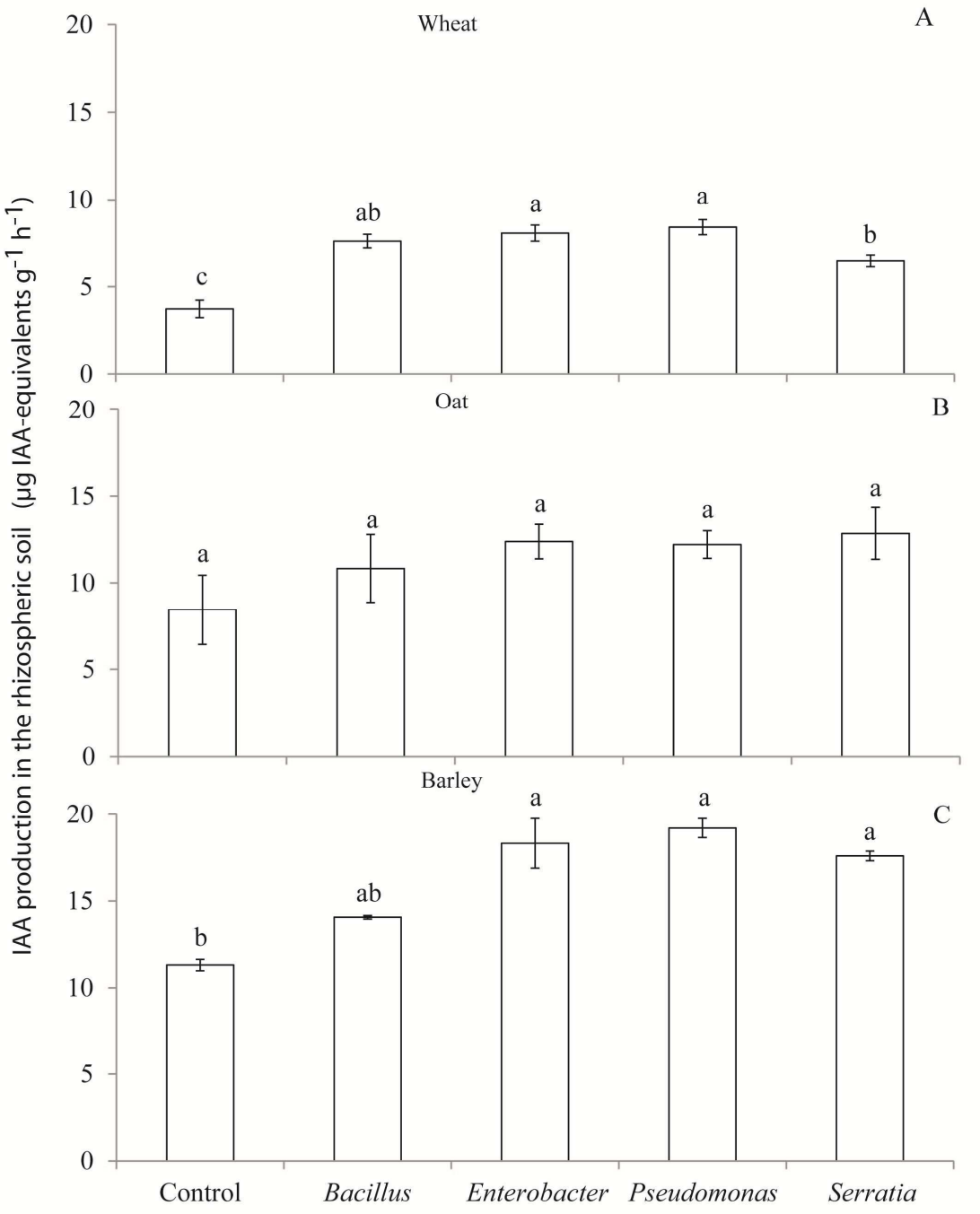

Figure 4.2. Potential of indole acetic acid production in rhizosphere soil from inoculated plants of A) wheat, B) oat and C) barley. Values represent mean ± standard error (average of three repeats). Different letters denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Student's t-test using JMP).

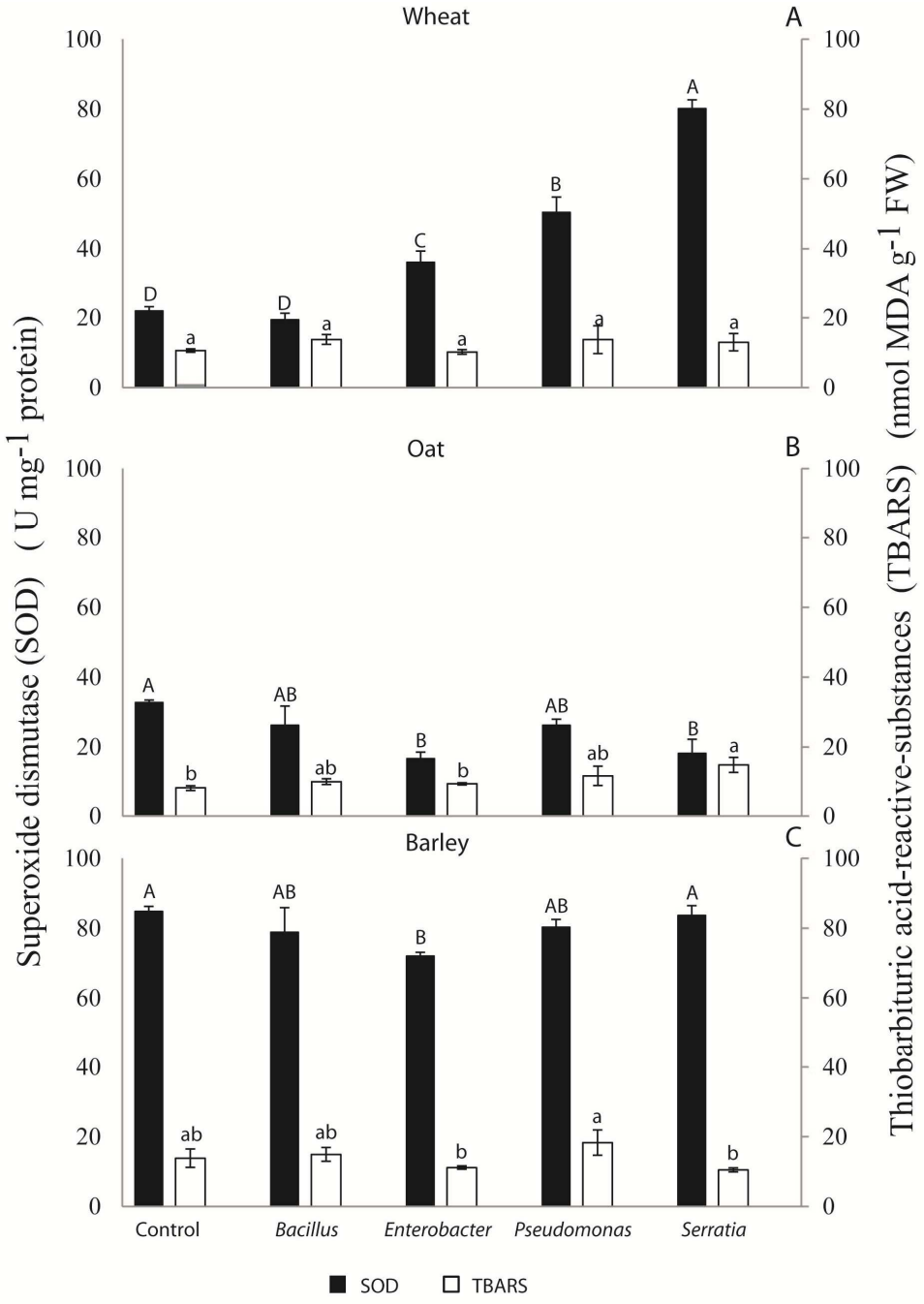

Figure 4.3. Root superoxide dismutase (SOD) and root TBARS activity from inoculated plants of A) wheat, B) oat and C) barley. For each parameter, means (±standard error). Different uppercase letters indicate differences (P<0.05, comparisons of means were carried out for each pair with Student's t-test using JMP) between treatments respect to SOD. Different lowercase letters indicate statistically significant differences (P<0.05, comparisons of means were carried out for each pair with Student's t-test using JMP) between treatments respect to TBARS.

Bacterial community composition in rhizosphere

The PCR-DGGE profile of the 16S rRNA gene is shown in Figure 4.4. Despite that the presence of some dominant bands were observed in wheat plants inoculated with *Enterobacter* sp. N0-29PA and *Pseudomonas* sp. N1-55PA, in general terms consistent differences between bacterial community structures among the treatments was not produced by rhizobacterial inoculation. Cluster analysis based on the DGGE profiles showed high similarity (> 90% in wheat, > 95% in oat, and >86% in barley) in banding pattern, suggeting that the inoculation had little effect on the soil rhibacterial diversity.

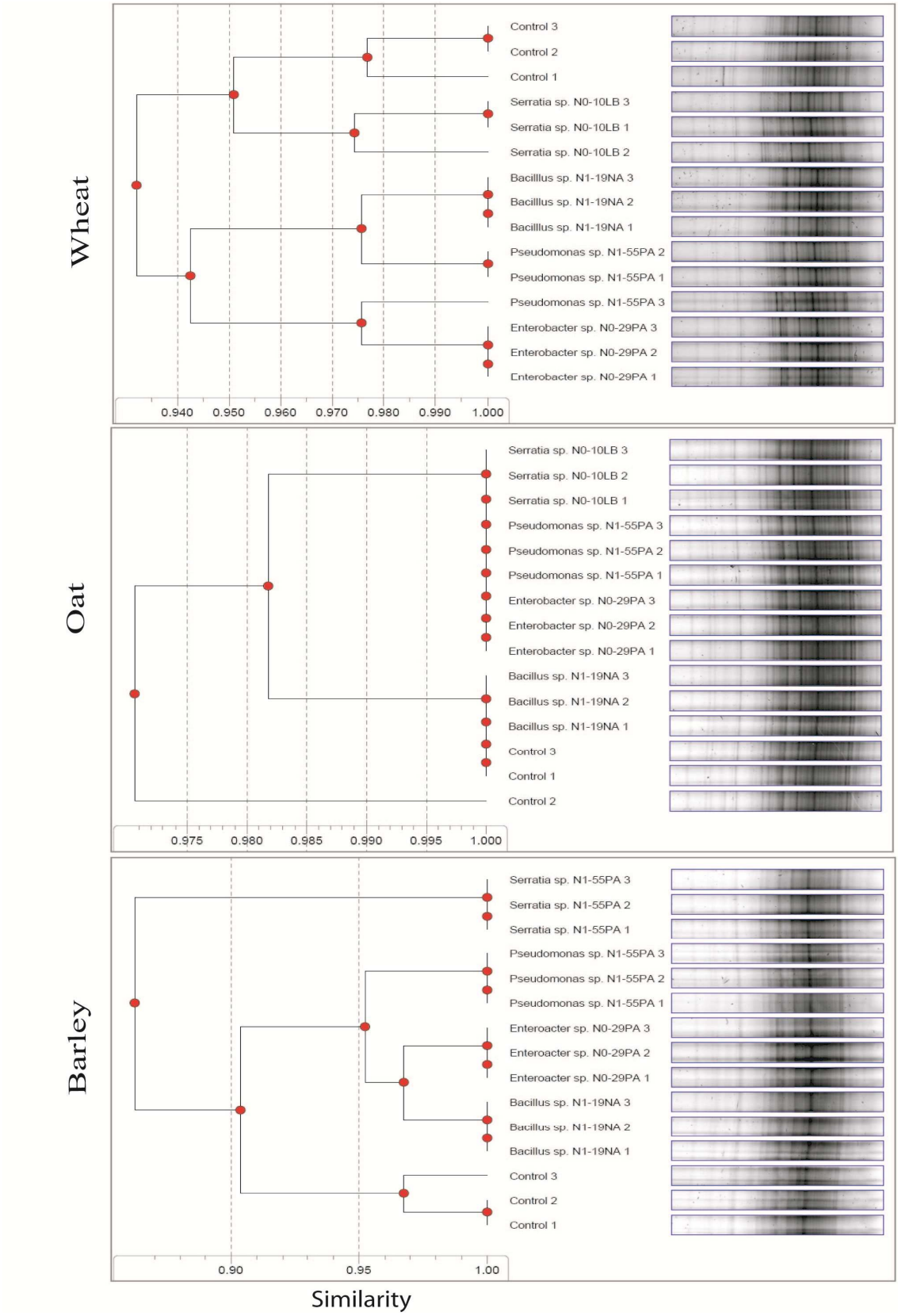

Figure 4.4. Dendrogram of denaturing gradient gel electrophoresis (DGGE) profiles of rhizobacterial communities by cluster analysis.

4.4. **Discussion**

Since the discovery of PGPR, many studies have demonstrated the beneficial interactions between PGPR and plants (Dardanelli et al. 2010). One of the greatest remaining challenges for better managing these bacteria, or for development of specific strains for use as biofertilizers, is the development of methods to monitor their population and activity in soils (Arora et al. 2010). There is also a basic lack of understanding of the contribution of the individual functional traits that are involved in plant growth promotion, and their mode of action in affecting plant physiological processes. Rhizobacteria were originally isolated and described based on their ability to solubilize P (Garretsen 1948). Subsequently, the plant growth promotion conferred by many of these bacteria was shown to involve IAA production. The isolation of PGPR on the basis of their ability to solubilize P was thus a fortuitious screening procedure that was able to cultivate PGPR. Likewise, the early use of *Azospirillum* was for fixation of nitrogen, and only later it was shown that the amounts of nitrogen fixed by these bacteria was of little relevance for improving plant growth as compared to their ability to stimulate root growth via the production of IAA (Okon and Kapulnik 1986; Spaepen et al. 2008). Today most PGPR are recognized to have multifunctional characteristics including production of growth hormones, suppression of ethylene, mobilization of iron, N-fixation, production of antibiotics, and production or degradation of HCN in the plant rhizosphere. Many PGPR also produce antibiotics and some can induce the systemic resistance of plants to improve disease tolerance.

In this research, experiments were conducted to examine some of the multifunctional properties of PGPR and the degree to which these are affected by interactions with a specific plant host. Experiments compared four strains of

rhizobacteria on three cereal plant species in a single soil type that was deficient in available P, but that contained high levels of total inorganic and organic P. This soil was selected as a model for P limiting soils in southern Chile, where grain crops are often P limited and require high applications of fertilizers to sustain their productivity. Thus much of the focus of this research was on the P solubilization and P mineralization abilities of the selected strains and their ability to mobilize P. In general terms, this study revealed that inoculation of selected rhizobacteria strains did not consistently enhance the biomass, P-uptake and P concentration of cereal plants. It is well known that the efficacy of inoculation is influenced by competition by the indigenous microbial community (Bashan 1998). Of the all possible combinations of plants and inoculants in experiment I, there were only two instances when inoculants (*Bacillus* sp. N1-19NA and *Enterobacter* sp. N0-29PA) improved the P concentration in wheat. This effect was only significant when the inoculants were introduced into sterilized soils, and lead to an approximate 7% increase in P concentrations in the plant tissues. A similar trend was observed in nonsterile soil, but the effect was not significant. In the nonsterile soil, the level of variation among replicates was 2-5 times greater than in sterile soils, suggesting that the inconsistency was due to competition with the native microbial community. These results are similar to those reported in previously published studies. Krey et al. (2011) reported no significant effects on the plant growth by the application of two PGPR strains (*Pseudomonas fluorescens* DR54 and *Enterobacter radicincitans* DSM 16656) alone and in combination with organic fertilization (cattle manure and biowaste compost) on growth of maize and oilseed rape. In the same way, P concentration of shoot and root was not increased by rhizobacterial inoculation under low P availability condition in both greenhouse experiments. In a similar study, Ramírez and Kloepper (2010) showed data where in the control (soil available P 3 mg kg^{-1}) was not observed

an effect on shoot P content of Chinese cabbage by *Bacillus amyloliquefaciens* FZB45 inoculation. Fernández et al. (2007) observed that the P content of soybean plants was same between plants inoculated with several phosphate solubilizing bacteria (*Pseudomonas fluorescens*, *Enterobacter* sp. *Burkholderia* sp. and *Bradyrhizobium* sp.) and soybean plants uninoculated. Also, Malboobi et al. (2009) also reported that different phosphate solubilizing bacteria had no significant effects on P concentrations of potato leaves at the tuber stage in the high- and medium-P inorganic soil types.

It is noteworthy, *Enterobacter* N0-29PA significantly increased the P concentration in sterile soil in wheat under P basal fertilization (greenhouse experiment I). This bacterium also increased the plant biomass and P-uptake of oat plants without P-fertilization (greenhouse experiment II). The results further suggested that this mobilization of P by *Enterobacter* sp. N0-29PA may be related to its production of extracellular phytase (data not shown) allowing it access to the organic P in this soil, which contained 993 mg kg^{-1}.P concentrations were increased approximately by 100 mg kg^{-1}, irrespective of fertilization. These results are in agreement with previous reports that *Enterobacter* strains produce extracellular phytase (Yoon et al. 1996; Konietzny and Greiner 2002). Bacterial phytase are actively secreted into soil, where they participate both in decomposition of plant debris and in the liberation of P from soil organic compounds that must be dephosphorylated, after which the liberated P can be taken up by plants (Richardson and Simpson 2011).

The possible effect of increased IAA-producing phosphobacteria on P availability was further evaluated with respect to extractable P using the Olsen extraction method. In both cases where the two effective strains were shown to improve plant P concentrations, the levels of extractable P were significantly elevated in soils inoculated with either *Bacillus* sp. N1-19NA or *Enterobacter* sp. N0-29PA as compared

to soils inoculated with the other two test strains or the uninoculated control soil. The mobilization of P is likely caused by induction of phytase enzyme activity to supply P for the bacteria that are actively growing in the plant rhizosphere. At elevated enzyme activity levels, the bacteria may liberate P that can be taken up by the plant before it is immobilized by the bacteria. Or as postulated by Marschner et al. (2011), the P that is immobilized into the bacterial cells may be release during death and turnover of the bacteria along the older root parts behind the root apices. Bacterial phospholipids contained in the cell membranes of bacteria are highly labile and undergo dephosphorylation soon after the death of bacterial cells. Prior research has similarly shown that extractable P levels can be increased after soil inoculation, but have not investigated the mechanism by which this occurs. Sundara et al. (2002) showed an increase in plant (sugarcane) available P status in the soil upon addition of *Bacillus megaterium*. Similar observations have been reported by Zaidi et al. (2004), where the available P status of the soil was improved by the addition of *Pseudomonas striata*. Still other processes by which bacteria might enhance plant P uptake could involve stimulation of root hair formation, or increased rates of exudation by plant roots. IAA production by bacteria stimulates cell division and expansion of the tissues in the zone of elongation where the majority of root exudation occurs. Organic acids contained in the root exudates in turn may desorb P from mineral surfaces, or complex mineral P. Root exudates are also involved in priming of organic matter mineralization, leading to enhance microbial activity and mineralization of both N and P contained in soil organic matter.

One of the curious results from this research was the observation that root surface phosphatase activity was decreased such that there was an inverse relationship between the level of extractable P and the phosphatase activity of the roots. This was

observed in soils inoculated with both *Bacillus* sp. N1-19NA and *Enterobacter* sp. N0-29PA, the only two strains that significantly increased P uptake by the plant. While difficult to explain, this phenomenon was also reported by Paredes et al. (2011), in which they found that the root surface phosphatase was inhibited at least 65% in response to increase soil available P. In the research conducted here, P concentrations in the inoculated plants were measured at 800 mg kg^{-1}, indicating the plants were still very P deficient (Sanchez 2007) and in which case the plants would be hypothesized to produce phosphatases in order to increase P mobilization and uptake. The suppression of phosphatase activity suggests that this enzyme is not regulated in relation to P availability. The degree to which root surface phosphatase activity is regulated by P availability is not yet well understood. On the other hand, the suppression of this enzyme in the presence of bacteria that putatively enhance P availability requires further explanation. One possibility is that local elevated P concentrations in the soil may suppress the induction of the surface enzyme activity even while the plant shoot suffers from P deficiency.

On the other hands, *Pseudomonas* N1-55PA inoculation increased acid phosphatase and urease in oat. Mäder et al. (2011) reported that the application combined of PGPR (fluorescent *Pseudomonas* strains: *P. jessenii* R62 and *P. synxantha* R81) and arbuscular mycorrhizal fungi increased soil enzyme activities of alkaline phosphatase, acid phosphatase, urease and dehydrogenase in wheat rhizosphere. Also, it has been reported that inoculation with *Bacillus subtilis* and *Enterobacter agglomerans* significantly increased the urease and phosphatase activities of the rhizosphere soil in lettuce and tomato plants, respectively (Kim et al. 1998; Kohler et al. 2007).

Along with enzyme activities, other factors may interact to influence P availability and uptake by plants. Production of IAA by PGPR has been suggested as

one of the most important factors affecting plant growth and may indirectly affect P-uptake as described above, by increasing root hair production or by altering plant root exudation (Lebuhn et al. 1994; Marschner et al. 2011). Numerous studies have shown an improvement in plant growth and development in response to seed or root inoculation with various microbial inoculants. As was expected, results from the current study showed that plants that were inoculated with IAA-producing phosphobacteria strains enhanced the potential production of IAA in soils amended with the tryptophan. This amino acid is a precursor of IAA and is a component of root exudates. When secreted into the rhizosphere, bacteria can produce IAA that is taken up by the plant, causing root growth and proliferation of root hairs (Cassán et al. 2011). The mode of action for increasing root growth is thought to involve an auxin mediated decrease in the pH of the cell walls, which enable the cellulose microfibrils to slip past one another and allow the root cells to expand (Hopkins and Hüner 2009). Subsequently, the cell walls are fixed by displacement of protons with divalent calcium, which stabilizes the cell walls from further expansion. The regulation of auxin and root elongation is further mediated by interactions with ethylene, which can be influenced by bacteria that produce the enzyme ACC deaminase. This enzyme degrades the precursor of ethylene. This research did not examine ACC activity of the soil inoculants, and this remains as an open question for further study.

In this research, studies were conducted to examine the relationship between plant P status and plant stress as indicated by lipid peroxidation to the plant membranes (revelated by TBARS). Previous work has suggested that P deficiency results in elevated superoxide dismutase (SOD) activity of the plant roots, and reflects a response to the generation of reactive oxygen species (ROS) caused by P deficiency (Juszczuka et al. 2001; Shin et al. 2005; Tewari et al. 2007). However, this is a relatively unstudied

topic in cereal plants. Here, we examined the hypothesis that inoculation with beneficial IAA-producing phosphobacteria may stimulate SOD activity. Together the SOD and TBAR activity measurements can be used to assess the effects of PGPR on stress caused by plant P deficiency. Results shown in Figure 4.3 showed erratic effects of inoculation on these enzyme activities. Three of the inoculants (*Enterobacter* sp.N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB) stimulated SOD activity in wheat, but suppressed this activity in oat, and had no significant effect on barley.

Another possibility unrelated to the P hypothesis is that the changes in activity of SOD and enzymes associated with lipid peroxidation (TBARS) were instead associated with other functions of the PGPR related to the inducible systemic resistance response that can be conferred by some PGPR (Dimkpa et al. 2009). ROS are continuously produced as a result of aerobic metabolism or in response to biotic and abiotic stresses. The SOD enzymes, ROS scavenging, are very important in amelioration the damage caused by oxidative stress in plants (Raychaudhuri and Deng 2000; Alscher et al. 2002; Gusta et al. 2009). In this context, the activity of SOD has been reported to increase under diverse stress situations such as drought, chilling, metal toxicity, and disease. Increases in SOD activity are often correlated with the tolerance of the plant against abiotic stresses (Raychaudhuri and Deng 2000; Tamás et al. 2004; Cartes et al. 2012), and is also associated with induced systemic resistance (ISR) in plants (Jetiyanon 2007; Li et al. 2008; Liang et al. 2011). The results of the research conducted here with respect to the effects of PGPR on this aspect of plant physiology represent a preliminary investigation of this topic and the possible interactions between plant P status and the induction of the systemic resistance response.

One of the most difficult questions in the development of PGPR soil inoculants is their interaction with the native microbial community. PGPR that are introduced into

soils must compete with indigenous rhizobacteria that are well adapted to the local environment, and may be influenced by a wide range of signal molecules secreted by plant roots or by other microorganisms. In this research, experiments were conducted to examine possible broad changes in the species composition of the rhizosphere communities using PCR-DGGE of 16S rRNA genes. The technique is low resolution in that only a small number of bands (<100) represent many thousands of species that are present in the soil (Winding et al. 2005). For individual microorganisms to be represented in the DNA band profiles, they should comprise at least 1% of the total DNA (MacNaughton et al. 1999; Casamayor et al. 2000). Thus this technique resolves only very broad changes in community structure. In previous research, this technique has been employed to follow changes in the population size of soil inoculants, but as individual PGPR strains rarely exceed 10^5 cell per gram in communities that contain 10^9 or more cells per gram soil, it may not be possible to track the inoculants (Bergsma-Vlami et al. 2005; Winding et al. 2005). In this research, the DGGE gels did not reveal consistent differences between bacterial community structures among the treatments. This is in agreement with a prior study by Herschkovitz et al. (2005) that reported that *A. brasilense* inoculation did not alter or disrupt the microbial structure at the group-specific level in maize rhizosphere as revealed by DGGE fingerprints analysis. Likewise, Lerner et al. (2006) reported that no prominent effect of *A. brasilense* inoculation was observed on the bacterial communities of plant roots grown in two different soils by DGGE and ARISA (automated ribosomal intergenic spacer analysis). Piromyou et al. (2011) inoculated forage corn rhizosphere with *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 and they reported that dominant species in microbial community structure were not interfered by PGPR strains.

4.5. **Conclusions**

In general, plant response to rhizobacteria inoculation was variable depending of the inoculated strain and cereal plant species assayed. However, the rhizobacteria inoculation clearly influenced parameters related to plant biomass, phosphorus uptake, rhizospheric enzymatic activities, such as indole acetic acid potential, root surface phosphatase, acid phosphatase, urease and phosphorus availability. In the present work, only *Enterobacter* N0-29PA inoculation did improve the plant biomass, P uptake and P concentration in cereal plants compared with uninoculated control. *Enterobacter* N0-29PA significantly increased the P concentration in wheat plant grown in sterile soil with basal P fertilization, and plant dry biomass and P uptake of oat plants grown in non-sterile soil without P fertilization. DGGE fingerprint analysis revealed that plant inoculation with rhizobacteria had slight effect on the rhizosphere bacterial communities among treatments in each of the cereal species. In contrast, the inoculation with rhizobacteria produced changes on acid phosphatase and urease activities in the rhizosphere and increased the potential of IAA production in the rhizosphere. Interesting, the inoculation with *Enterobacter* sp. N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB increased the antioxidant root enzyme superoxide dismutase in wheat plants, paremeter related with plant defense mechanisms.

This study show that plant growth promotion potential of the rhizobacterial strains requires major evaluation to be implemented in phosphorus-deficient Andisols. Moreover, the response of cereal crops to the application of these rhizobacterial strains in appropriate combinations with chemical and/or organic phosphorus fertilizers could be considered for future experiments.

4.6. **References**

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Chapter 5

Global Discussion and General Conclusions

Global Discussion

Phosphorus (P) is one of the major essential macronutrients for biological growth and development. In southern of Chile, up to 43% of total agricultural land is acid and moderately acid volcanic ash-derived soils (Andisol). Andisols contain large reserves of total P, a part of the accumulated P depends on regular application of chemical fertilizers or organic amendments. But P fixation and precipitation with soil constituents cause a major P-deficiency and severely restrict the growth and yield of crop plants. Modern agriculture is heavily depending on the application of chemical inputs particularly, P fertilizers. This practice is, however, not sustainable and also uneconomic, producing also the continuous accumulation of P in Andisols. In order to raise the availability of P and to reduce the use of chemical fertilizers, solubilization of insoluble inorganic P and mineralization of organic P by beneficial rhizospheric phosphobacteria has provided an alternative to chemical phosphate fertilizer.

Beneficial free-living rhizosphere bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR) and are found in association with the root surfaces of many different crop plants. The PGPR are capable of facilitating plant growth after inoculation onto seeds or when already present in rhizospheric soils in high concentration. The PGPR facilitate plant growth by synthesizing or altering the concentration of phytohormones (IAA), disease suppression (siderophores, antibiotics and cyanide), and solubilization of mineral phosphates and other nutrients and by increase stress plant tolerance. And hence, use of such PGPR may be a viable alternative to chemical fertilizers for increasing the productivity of various crops in volcanic soils. However, despite their proven ability of growth promotion, PGPR have

yet to fulfill their promise and potential as commercial bioinoculants. Understanding functional diversity of PGPR is vital for sustainable production in agroecosystems.

In this thesis were isolated and selected native rhizobacteria capable of mobilizing insoluble forms (organic and inorganic) of phosphorus (P) and synthesize indole acetic acid (IAA). The effects of long-term nitrogen (N) fertilisation on the occurrence of potential culturable PGPR in rhizosphere soils from pastures grown in Chilean Andisols was examined. In vitro the effects of organic acids (citric, malic and oxalic acids), metals (Al and Mn) and N supply (urea and ammonium sulphate) on indole acetic acid (IAA) production and phosphorus (P) liberation by selected strains was also evaluated. The most efficient culturable IAA-producing phosphobacteria (IAAP) were identified as *Bacillus* sp. N1-19NA, *Enterobacter* sp.N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB. In this study was demonstrate that N fertilisation and factors present in Chilean Andisols have a relevant role in the occurrence and performance of culturable rhizobacteria containing beneficial traits for plant growth. Furthermore, this influence should be taken into account when microbial inoculants are developed for applying them in volcanic acidic soils.

Latter, the influence of selected rhizobacteria on cereal plants and rhizosphere parameters were evaluated and the results showed differential responses of cereal species to rhizobacteria inoculation respect to plant growth, plant P uptake and soil biological activities under P-deficient soil. Interestlty, the inoculation of *Enterobacter* sp. N0-29PA, increased plant dry biomass and P uptake in oat plants. In general, these results showed that under P-deficient conditions, the rhizobacteria inoculation not only can affect the plant growth, P uptake and P concentration of cereal plants but also can affect other relevant as enzymatic rhizosphere activity and plant defense mechanisms agaisnt stress.

Several isolates of *Enterobacter* have been developed as biofertilizers. However, to be used as successful biofertilizers a greater comprehensive knowledge of their ecology is desired. An understanding of the mechanisms enabling this bacterium to interact with cereal plants will be essential to fully achieve the biotechnological potential of efficient plant–bacterial partnerships for a range of agricultural applications. In addition, future research is required to understand the performance of *Enterobacter* sp.N0-29PA under volcanic soil agricultural conditions and their effects on efficiency of P fertilization utilization and P uptake.

General Conclusions

Considering that the two main goals of this study were: (1) to examine the potential for enhancing plant growth by native rhizobacteria with PGPR activities and how the environmental factors influence the performance the these PGPR activities, and (2) the inoculation of cereal plants with selected native indole acetic acid (IAA)-producing phosphobacteria under P available deficient soil. We can conclude that in pasture growing in volcanic soils there are native rhizobacteria with various PGPR activities which are suitable for use as potential crop inoculants. But, we must to consider that nitrogen fertilization management change culturable phosphobacteria community profile in the rhizosphere because acidification of soil. The typical Al and Mn content in volcanic acid soil influence IAA production and bacterial growth. Furthermore, our studies of the different organic acids like citric, malic and oxalic, for simulating plant roots exudates in the rhizosphere, produced changes in bacterial growth and their IAA biosynthesis performance. Nevertheless, the impact of these factors is strain dependent. The inoculation of cereals plant with selected strains *Bacillus* sp. N1-19NA, *Enterobacter* sp.N0-29PA, *Pseudomonas* sp. N1-55PA and *Serratia* sp. N0-10LB influenced plant and rhizospheric parameters but not rhizobacterial community structure evaluated by denaturing gradient gel electrophoresis (DGGE). Although, the changes in the activity of the rhizosphere and plant parameters can be attributed to inoculation. We concluded that the bacterial community structure evaluated by DGGE after rhizosphere inoculation is not a methodology appropriate for detecting changes in rhizobacterial communities. The results of the inoculation wheat, oats and barley showed that not all selected native IAA-producing phosphobacteria have the ability to promote the plant

growth and phosphorus uptake under conditions of deficient in available P soil. But, the inoculation with native strain *Enterobacter* N0-29PA showed promising results in relation to increase plant biomass and phosphorus uptake in cereal plants.

Suggestions for future research

- The future research should be include toward the detection by molecular techniques genes involved in the solubilization of inorganic phosphate, mineralization of organic phosphate and indole acetic acid production.
- Future efforts aimed at tracking PGPR populations and their activity in the rhizosphere may benefit from the use of reporter gene constructs such as those using fluorescent proteins that are coupled to the promoters for relevant PGPR functional genes. The activity and population of PGPR will likely vary along the root axis, and will be very different than that for cells located in the bulk soil that is not under the influence of the plant roots. In studies of the ecology of PGPR it is especially important to consider microsites where activity is relevant to particular processes such as phosphorus mobilization or production of hormones, or expression of enzymes that are involved in nutrient mineralization and other processes that affect plant growth.
- New screening strategies should be used to isolation of a wider range of rhizobacterial species with PGPR activities.
- Field evaluations during several grown seasons of plant growth are required to determine the results under agronomic conditions.

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Annex

Thesis publications

Martínez OA, Jorquera MA, Crowley DE, Mora ML (2011) Influence of nitrogen fertilisation on pasture culturable rhizobacteria occurrence and the role of environmental factors on their potential PGPR activities. Biol Fertil Soils 47: 875–885

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