Exploring the Relation Between Plant Phosphorus Nutrition and Growth Promotion by *Bacillus subtilis/amyloliquefaciens* Strains

by

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Abstract

The use of plant growth-promoting rhizobacteria (PGPR) as biofertilizers is of increasing interest due to economic and environmental issues. Phosphorus (P) is one of the most limiting plant nutrients because of its reduced availability in soil and the limitation of its sources for fertilization. Because of this, PGPR are considered as a potential tool to reduce chemical P fertilization, either by increasing the access to normally unavailable P forms in soil or by increasing the efficiency of plant P uptake. Most of the PGPR-based products commercially available contain strains of aerobic endospore-forming bacteria, also referred to as bacilli, because of their capacity to be formulated in economic and long shelf-life products. However, inconsistent results remain as a major limitation for wide spread use of PGPR. The present work aimed at facing this problem by studying the relation between bacilli PGPR and plant P nutrition within a conceptual framework that considers plant growth promotion as a three-party interaction: host, PGPR strain, and environment (soil). In this context, the first chapter presents a literature review about the state-of-the-art knowledge on the mechanisms of action of bacilli PGPR. Here, particular attention is given to the agronomical relevance of the mechanisms described so far by analyzing the techniques and experimental systems used. The analysis of the techniques used allows determination of the presence of causal effects, while the experimental systems determine the validity of results to understanding the processes in nature. In the second chapter, two factors, one involved in the PGPR strain – soil interaction (soil P content) and the

other in the PGPR strain – host interaction (inoculum concentration), were evaluated for their effect on early plant growth promotion mediated by P-solubilization. This study used a soil-plant experimental model consisting of Chinese cabbage and the phytase-producing strain *Bacillus amyloliquefaciens* FZB45. In the third chapter, a molecular study was conducted to explore the relation of phytase-coding DNA sequences and taxonomy in the *B. subtilis/amyloliquefaciens* group and the usefulness of this gene for discrimination between those two species. Finally, the fourth chapter explored the relation between PGPR-induced changes of root architecture and plant P uptake. For this, 73 bacilli strains were screened for production of key molecules involved in alteration of root architecture, and 4 of them were tested for their effect on root architecture of Chinese cabbage. In addition, the combined effects of *B. amyloliquefaciens* FZB42 and different soil P levels on root architecture and P uptake of Chinese cabbage were tested in a soil-plant model system.

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Chapter 1. Mechanisms of Action in Bacilli-PGPR: Basic Knowledge for Practical Use

1. Introduction

Bacterization, the treatment of seeds or seedling roots with cultures of bacteria to improve plant growth, has been studied for several decades (Brown, 1974). In 1978 the term *plant growth-promoting rhizobacteria* (PGPR) was introduced by Kloepper and Schroth (1978) to refer to those beneficial bacteria that aggressively colonize plant roots (Kloepper, 1993; Schroth and Becker, 1990)[1]. Benefits produced by PGPR have been widely documented on different plant species including crops, ornamentals, vegetables, and forestry (Zahir et al., 2004). These beneficial effects include increases in a number of parameters such as the rate of seedling emergence,, growth of roots and shoots, yield, leaf area, chlorophyll content, concentration of nutrients, as well as inducing delayed leaf senescence and tolerance to drought and other stressfactors (Lucy et al., 2004). These benefits of PGPR are advantageous not only for agriculture but also for environmental purposes such as plant-mediated bioremediation (reviewed by Zhuang et al., 2007) and revegetation of eroded lands (reviewed by Bashan et al., 2008).

[1] For this review, symbiotic nitrogen-fixing bacteria, *i.e.* rhizobia, are not considered as PGPR.

Much attention has also been given to the use of PGPR as biological control agents for plant diseases, a topic that has been reviewed several times (Compant et al., 2005; Haas and Défago, 2005; Someya and Akutsu, 2006). Despite the research conducted during the last three decades, to date, there has only been limited use of PGPR as alternatives for chemical fertilizers and pesticides in agricultural production systems (Banerjee et al., 2006; Fravel, 2005; Siddiqui, 2006). This situation has been attributed to two major limiting factors: the limited number of formulations commercially available and the inconsistent performance of these formulations (Emmert and Handelsman, 1999; Siddiqui, 2006). While the latter factor remains as an obstacle, the former, the availability of commercial formulations, has been partially overcome by the use of strains of aerobic endospore-forming bacteria (AEFB) (Emmert and Handelsman, 1999; Kloepper et al., 2004). Easy mass production and the unique formation of highly resistant endospores make bacilli rather feasible to be formulated (Mathre et al., 1999; Ongena and Jacques, 2008). These advantages have made possible the development of products with a shelf-life of over one year, which is generally required for successful integration into the agricultural market (Driks, 2004; Kloepper et al., 2004).

The variability in results obtained with inoculation is commonly considered as the main limiting factor for PGPR implementation in agriculture (Siddiqui, 2006; Vessey, 2003). This variability is thought to be mainly caused by differences in soil properties, but plant-associated factors and agronomic practices are also considered to play a role (Ownley et al., 2003; Richardson et al., 2001b). One way to overcome this inconsistency is to understand the mechanisms by which PGPR exert their benefits. The knowledge of these mechanisms under natural environments should make it possible to meet the conditions for optimal results and to

predict the outcome (Niranjan Raj et al., 2006). However, the mechanisms of action of PGPR are yet to be fully understood. Important insights have revealed several ways by which PGPR can benefit plants, a very active topic in PGPR research (Compant et al., 2005; Glick et al., 1999). Nevertheless, little is known about these mechanisms under natural conditions due to the fact that most of the experiments leading to these findings have been based on artificial systems. Agricultural systems include several components that will determine the PGPR performance. Soil, for instance, affects not only plant growth but also the overall bacterial physiology, which confers on bacterial strains a very different behavior from that seen in artificial media (Vilain et al., 2006).

This review aims to summarize current knowledge on the mechanisms of action in bacilli-PGPR[2], which represent most of the PGPR-based products commercially available in North America and Europe. Special attention is given to the agronomic relevance of the mechanisms described so far, pointing out some aspects that should be considered to make this knowledge significant for agriculture. This perspective is based on the thought, previously stated by Emmert and Handelsman (1999), that detailed studies of the whole system are needed to have sufficient insight that allow us to avoid failures and reduce variability. Also, the attention is brought to the nature of the evidence supporting the putative mechanism in the cited studies, trying to classify this evidence as direct or indirect. Finally, some considerations are made regarding the role of ecology and genetic engineering for improving the performance of bacilli inoculants.

[2] In this review the terms "bacilli" and "aerobic endospore-forming bacteria" (AEFB) will be used as synonyms to mean *Bacillus* spp. and closest relatives.

2. Mechanisms of Action in Bacilli-PGPR

The ways by which PGPR produce their beneficial effects have usually been classified in two different groups: direct and indirect mechanisms (Glick et al., 1999). Direct mechanisms are those in which rhizobacteria increase the plant growth by producing plant growth regulators or solubilizing minerals; whereas indirect mechanisms relate to plant growth promotion as a result of decreasing deleterious effects of plant pathogens (Cattelan et al., 1999).

So far, the majority of research on mechanisms has been done with *Azospirillum* spp. (Bashan et al., 2004) and *Pseudomonas* spp. (Haas and Défago, 2005). The experimental approaches used are diverse, but the most compelling evidence has been obtained by the use of techniques in bacterial genetics, such as mutant analysis, cloning, and sequencing. For bacilli, in contrast, research specifically regarding PGPR-traits is limited. Many of the underlying mechanisms generally attributed to bacilli PGPR strains are just an extension of the research conducted with the other two mentioned genera. Intriguingly, this has occurred in spite of the fact that *Bacillus subtilis* has been used as a model for genetic and biochemical research on gram-positive AEFB for several decades (Sonenshein et al., 2002; Wipat and Harwood, 1999). Nevertheless, it is well established that certain bacilli strains benefit plants by both groups of mechanisms, *i.e.*, promoting plant growth and/or displaying biocontrol. New papers supporting this are consistently published and thorough reviews summarizing the effect of bacilli-PGPR for plant growth promotion (Kloepper et al., 2004; McSpadden-Gardener, 2010) and disease control

(Emmert and Handelsman, 1999; Ongena and Jacques, 2008; Shoda, 2000)[3] are available. For this reason, the present review does not discuss the effectiveness of bacilli PGPR, but it only focuses on the mechanisms of action behind their benefits.

The literature on bacilli PGPR includes many different strains and species, most of which belong to the genus *Bacillus* or to the closely related genus *Paenibacillus*. From the plethora of isolates reported in the literature, certain strains clearly stand out because of the number of studies conducted with them, the level of detail in their characterization, and because some of them are commercially available (Table 1.1). These are the strains most commonly used in the PGPR research; therefore, the state-of-the-art knowledge on AEFB as PGPR is mainly based on results obtained with this group of strains.

This research has revealed enlightening clues about the mechanisms of action in bacilli PGPR. However, many gaps are still to be filled and, in many cases, the details behind the beneficial effects are completely unknown. In the following section, each of the mechanisms of action proposed so far is discussed. This discussion begins with the indirect mechanisms given that the majority of the PGPR research with bacilli strains has focused on their use for biological control (Ongena and Jacques, 2008; Shoda, 2000). Thus, the experimental approaches classically taken, as well as the advantages and limitations of these approaches, are illustrated in the knowledge available on this group of mechanisms. Then, the direct mechanisms are covered, emphasizing also the nature of the evidence and the challenges for practical use.

[3] These reviews discuss the control of both foliar and soilborne diseases. Because the present review is focused on root-colonizing bacilli, only biocontrol of soilborne pathogens is considered, except when a reduction of a foliar disease is attributed to induced systemic resistance (ISR).

Table 1.1 Outstanding bacilli-PGPR strains in the literature

Strain	Commercial Product	Beneficial Effect	Selected References
Bacillus subtilis GB03 [a]	BioYield®	PGP [e]/Biocontrol	Mahaffee and Backman (1993) Raupach and Kloepper (1998) Raupach and Kloepper (2000) Estevez de Jensen et al. (2002) Ryu et al. (2003a) Ryu et al. (2004)
Bacillus amyloliquefaciens IN937a [b]	BioYield®	PGP/Biocontrol	Raupach and Kloepper (1998) Raupach and Kloepper (2000) Zehnder et al. (2000) Ryu et al. (2003a) Ryu et al. (2004)
Bacillus pumilus INR-7 [c]	YieldShield®	PGP/Biocontrol	Wei et al. (1996) Zehnder et al. (1997) Zehnder et al. (2001)
Bacillus amyloliquefaciens FZB24	RhizoPlus® FZB24® (WG, fl., and TB)	PGP/Biocontrol	Krebs et al. (1998) Kilian et al. (2000) Idriss et al. (2002) Yao et al. (2006) Tahmatsidou et al. (2006)
Bacillus amyloliquefaciens FZB42 [d]	RhizoVital®42 fl.	PGP/Biocontrol	Krebs et al. (1998) Idriss et al. (2002) Koumoutsi et al. (2004) Chen et al. (2006) Koumoutsi et al. (2007) Chen et al. (2007a)
Bacillus amyloliquefaciens FZB45	Non commercialized	PGP	Krebs et al. (1998) Idriss et al. (2002) Makarewicz et al. (2006)
Bacillus subtilis MBI600	Subtilex®	Biocontrol/increased nodulation [f]	Knox et al. (2000) Knox et al. (2002) Estevez de Jensen et al. (2002) Knox et al. (2003)
Bacillus subtilis RB14	Non commercialized	Biocontrol	Ohno et al. (1995) Asaka and Shoda (1996) Asaka et al. (1996) Kondoh et al. (2000) Tsuge et al. (2001) Szczech and Shoda (2004) Szczech and Shoda (2005) Kita et al. (2005) Szczech and Shoda (2006) Rahman et al. (2006) Mizumoto et al. (2006) Mizumoto et al. (2007) Mizumoto and Shoda (2007)
Bacillus cereus UW85	Non commercialized	Biocontrol	Handelsman et al. (1990) Halverson and Handelsman (1991) Halverson et al. (1993) Silo-Suh et al. (1994) Stabb et al. (1994) Osburn et al. (1995) Milner et al. (1995) Milner et al. (1996a) Milner et al. (1996b) Emmert and Handelsman (1999) Dunn and Handelsman (1999) Dunn et al. (2003) Emmert et al. (2004) Peterson et al. (2006)
Paenibacillus polymyxa B2	Non commercialized	PGP/Biocontrol	Timmusk et al. (1999) Timmusk and Wagner (1999) Timmusk (2003) Timmusk et al. (2005) Selim et al. (2005)

[a] Also referred as GB122

[b] Also referred as GB99

[c] Also referred as GB34

[d] Only *Bacillus*-PGPR with whole genome sequence complete (Chen et al., 2007a)

[e] PGP: Plant growth promotion

[f] Improvement in nodulation by nitrogen-fixing bacteria

3. Indirect Mechanisms: Biocontrol

3.1. Antibiotics

3.1.1. *Bacillus* antibiotics

Antibiotics are defined as a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms that are deleterious to the growth or other metabolic activities of other microorganisms (Fravel, 1988; Raaijmakers et al., 2002). Antibiotic production is, by far, the most studied mechanism of biological control by PGPR (Fravel, 1988; Haas and Défago, 2005; Raaijmakers et al., 2002). The importance of this mechanism in bacilli is suggested by the fact that about 4% of the genome of *Bacillus subtilis* 168, a model strain for molecular studies, is devoted to antibiotic production (Kunst et al., 1997). In general, estimations say that, even though no strain produces all the antibiotics reported so far, 4-5% of this species' genome is occupied by antibiotic-related genes (Stein, 2005). In the case of *Bacillus amyloliquefaciens* FZB42, which is the first bacilli-PGPR strain with its complete genome sequenced, this percentage is even greater, reaching 8.5% (Chen et al., 2007a). This could suggest that, in beneficial AEFB, antibiotic production plays a central role, but the whole genome sequence of more bacilli-PGPR strains must be analyzed to make this conclusion.

To date, *B. subtilis* has been shown to produce more than two dozen antibiotics with a high diversity of structures and functions (Table 1.2) (Stein, 2005). For certain antibiotics, these functions go beyond antimicrobial action and have other biological properties, some of which are

Table 1.2 Classification and functions of the most studied antibiotics produced by *Bacillus subtilis* [a]

Chemical and Structural Classification			Main Functions	Antibiotic
Peptidic Compounds	Ribosomally synthesized	Type A-Lantibiotic	Inhibition of Gram-positive bacteria by forming voltage-dependent pores into the cytoplasmic membrane	Subtilin Ericin
		Type B-Lantibiotic	Inhibition of cell wall biosynthesis by complexing lipid II	Mersacidin
		Lantibiotic-like peptide	Inhibition of Gram-positive bacteria preferentially	Sublancin
		Lantibiotic-like peptide	Inhibition of a variety of Gram-positive bacteria	Subtilosin A
	Non-ribosomally synthesized	Iturin family -Cyclic lipopeptide-	Strong anti-fungal and haemolytic but only limited anti-bacterial activities - Biosurfactant properties	Iturin Bacillomycin Mycosubtilin
		Fengycin family -Cyclic lipopeptide-	Specific inhibition of filamentous fungi	Fengycin
		Cyclic lipopeptide	The most powerful biosurfactant known, detergent-like action on biological membranes, antifulgal, antiviral and antimycoplasma activities	Surfactin
		Trilactone macrocyclic ring	Siderophore-like action	Bacillibactin
		Dipeptide	Inhibition of a wide variety of bacteria and fungi	Bacilysin[b]
Non-peptidic compounds		Polyketide	Anti-bacterial activity[c,d]	Difficidin Bacillaene Macrolactin[d]
		Aminosugar	Inhibition of some Gram-positive and -negative bacteria	3,3'-Neotrehalosadiamine (NTD)
		Phospholipid	Selective inhibition of Gram-positive bacteria and nonfilamentous fungi[e]	Bacilysocin
		Phenolic derivative[f]	Anti-bacterial activities	Amicoumacin

[a]Table is based on the information given by Stein (2005)

[b] Hilton et al. (1988)

[c] Chen et al. (2006)

[d] Schneider et al. (2007)

[e]Tamehiro et al.(2002)

[f] Pinchuk et al. (2002)

relevant to plant-soil-microbe interactions. The best examples are lipopeptides and the phospholipid bacilysocin, which are surfactants with low molecular mass that are able to alter the physical and chemical properties at interfaces, affecting survival of the bacterial strain in soil and the rhizosphere (Stein, 2005). The lipopeptides surfactin and mycosubtilin, for instance, have been shown to play a role in surface motility and spreading *in vitro* (Kinsinger et al., 2005; Kinsinger et al., 2003; Leclère et al., 2006). In addition, several studies suggest that surfactin is required for pellicle and biofilm formation *in vitro* (Hofemeister et al., 2004; Kinsinger et al., 2005; Leclère et al., 2006) and *in planta* (Bais et al., 2004). Another example of the alternative biological role played by antibiotics is the ability of surfactin and fengycin to elicit induced systemic resistance (ISR). This was shown in bean and tomato against *Botrytis cinerea* where the response was accompanied by activation of key enzymes in the lipoxygenase pathway (Ongena et al., 2007).

3.1.2. Strategies to determine the role of antibiotic production by PGPR

The role of antibiotics in biological control has been of interest to researchers for a long time (Fravel, 1988). Raaijmakers et al. (2002) identified four lines of evidence classically followed to demonstrate the role and function of bacterially-produced antibiotics *in situ*. The first line of evidence is supported by the use of culture filtrates or purified antibiotics to provide similar levels of control as achieved by the producing wild-type strain. Secondly, inactivation of antibiotic production is conducted by mutagenesis. This should result in a reduced ability of the antagonistic bacteria to control the pathogen, and the original phenotype (biocontrol level) must be restored if mutants are complemented with the wild-type gene. The third line resorts to the

enhancement of antibiotic production in the producing wild-type strains, via introduction or modification of antibiotic biosynthetic or regulatory genes, in order to improve the biocontrol performance. Lastly, the fourth line of evidence uses the introduction of antibiotic biosynthetic genes in heterologous, non-producing strains and subsequent evaluation for their ability to control plant diseases. Similarly, Thomashow et al. (2002) described several commonly used experimental approaches to study antibiotic production by rhizosphere bacteria *in situ*. The first mentioned approaches are the use of chromatographic techniques and determination of the presence and activity of antibiotic genes for direct and indirect analysis, respectively. These authors consider that these experimental approaches give valuable information. However, they point out that the activity of antibiotics *in situ* will be influenced by factors such as stability and biological availability, which will vary according to the environmental conditions. Additionally, antibiotics can reach biologically significant threshold concentrations within microsites while remaining at low or undetectable levels overall. Based on this, Thomashow et al. (2002) stated that the use of genetically defined antibiotic deficient mutants is the best option to study the activity of a producer strain under different environmental conditions. The value of those mutants is based on the comparison with their wild-type strain, a procedure in which careful phenotypic screening is critical.

The experimental approach and test conditions used to assemble the evidence for a putative mechanism of action will determine the agronomic relevance of such evidence. Any of the commonly used approaches will give valuable information and, of course, the availability of tools and resources will determine which of those approaches should be taken. However, given that, in agronomic systems, PGPR are used as living cells applied to either soil, seed, or plant,

Table 1.3 *Bacillus* antibiotics with evidence supporting their role in plant protection

Antibiotic	Strain	Pathogen[a]	Test Condition	Experimental Approach	Reference
Iturin A	*B. subtilis* RB14	*Rhizoctonia solani*	Tomato (damping-off)	Knock-out mutant analysis	Asaka and Shoda (1996)
Bacillomycin D	*B. subtilis* AU195	*Alternaria solani* *Aspergillus flavus* *Botryosphaeria ribis* *Colletotrichum gloeosporioides* *Fusarium oxysporum* *Helminthosporium maydis* *Phomopsis gossypii* *Sclerotium rolfsii*	*in vitro*	Culture filtrates and purified compound	Moyne et al. (2004)
	B. amyloliquefaciens FZB42	*F. oxysporum* f. sp. *cucumerinum*	*in vitro*	Knock-out mutant analysis	Koumoutsi et al. (2004)
	B. subtilis B49	*Sclerotinia sclerotiorum* *Fusarium graminerum*	*in vitro*	Purified compound	Ramarathnam et al. (2007)
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*	Tomato (damping-off)	Overexpressing mutant analysis	Leclère et al. (2005)
		Botrytis cinerea	*in vitro*	Overexpressing mutant analysis	
		Fusarium oxysporum	*in vitro*	Overexpressing mutant analysis	
Fengycin	*B. subtilis* M4	*Pythium ultimum*	Bean (damping-off)	Culture filtrates and purified compound	Ongena et al. (2005)
		Botrytis cinerea	Apple (postharvest)		
	B. subtilis GA1	*Botrytis cinerea*	Apple (postharvest)	Culture filtrates and *in situ* detection	Toure et al. (2004)
	B. amyloliquefaciens FZB42	*F. oxysporum* f. sp. *cucumerinum*	*in vitro*	Knock-out mutant analysis	Koumoutsi et al. (2004)
	B. subtilis B49	*Sclerotinia sclerotiorum* *Fusarium graminerum*	*in vitro*	Purified compound	Ramarathnam et al. (2007)
	B. subtilis IB	*Fusarium graminearum*	*In vitro*	Purified compund	Wang et al. (2007)
Surfactin	*B. subtilis* RB14	*Rhizoctonia solani*	Tomato (damping-off)	Knock-out Mutant analysis	Asaka and Shoda (1996)
Difficidin /Oxydifficidin[a]	*B. amyloliquefaciens* FZB42	*Pectobacterium (Erwinia) carotovora*	Bioautography- *in vitro*	Knock-out mutant analysis – Purified compound	Chen et al. (2006)
		Erwinia amylovora	Apple (fire blight)	Knock-out mutant analysis	Chen et al. (2009b)
Bacillaene[a]	*B. amyloliquefaciens* FZB42	*Pectobacterium (Erwinia) carotovora*	Bioautography- *in vitro*	Knock-out mutant analysis – Purified compound	Chen et al. (2006)
Macrolactin[a]	*B. amyloliquefaciens* FZB42	*Pectobacterium (Erwinia) carotovora*	Bioautography- *in vitro*	Knock-out mutant analysis – Purified compound	Chen et al. (2006) Schneider et al. (2007)
Bacilysin[a]	*B. amyloliquefaciens* FZB42	*Erwinia amylovora*	Apple (fire blight)	Knock-out mutant analysis	Chen et al. (2009b)
Kanosamine	*B. cereus* UW85	*Phytophthora medicaginis*	Alfalfa (damping-off)	Knock-out mutant analysis	Silo-Suh et al. (1994) Milner et al. (1996a)
		Aspergillus flavus *Botrytis cinerea* *Ophiostoma ulmi* *Sclerotinia spp.* *Venturia inaequalis* *Rhizoctonia solani* *Alternaria* spp. *Colletotrichum* spp. *Fusarium* spp. *Helminthosporium* spp. *Verticillium* spp.	*in vitro*	Purified compound	Milner et al. (1996a)
Zwittermicin	*B. cereus* UW85	*Phytophthora medicaginis*	Alfalfa (damping-off)	Knock-out mutant analysis	Silo-Suh et al. (1994)

[a]Some foliar an fruit pathogens are included in the list to illustrate the biological potential *in planta* of the corresponding PGPR strains. However, only the control of soilborne pathogens is discussed in the text as the focus for the present review is on root-colonizing bacilli.

the use of the actual bacterial strain (wild-type and mutants) is much more conclusive than the use of culture filtrates or purified compounds. On the other hand, the test conditions define the environment in which the plant-PGPR-pathogen interaction takes place, which is critical for the performance of PGPR strains. In general, bacterial physiology undergoes profound changes in soil compared to its growth in laboratory media, a phenomenon that has been described for the soil saprophyte *Bacillus cereus* (Vilain et al., 2006). Similary, antibiotic production of *Bacillus amyloliquefaciens* CCMI changes during sporulation as well as antifungal activity is also altered under different nitrogen patterns (Caldeira et al., 2008). Similarly, production of the antibiotic kanosamine by *Bacillus cereus* UW85 is enhanced by the addition of ferric iron and suppressed by the addition of phosphate (Milner et al., 1996a). Another example is *Pseudomonas fluorescens* 2-79RN_{10}, whose ability to protect wheat against take-all disease was also greatly influenced by soil chemical properties, especially zinc and organic matter content (Ownley et al., 2003).

Most of the data on antibiosis were originally derived from assays performed *in vitro.* Therefore, assessing the impact of these antibiotics *in situ* is required for practical use of PGPR. The importance of this was illustrated with *Pseudomonas fluorescens* SS101 which produces the cyclic lipopeptide antibiotic massetolide A, a compound that inhibits the growth of *Pythium* spp. (Mazzola et al., 2007) and *Phytophthora infestans* (Tran et al., 2007) when applied pure. However, the inoculation of the non-producing mutant 10.24 reduced the infection by *Pythium* spp. in apple and wheat at the same level as the wild-type SS101, suggesting no role for this lipopeptide in biocontrol of the tested pathosystem (Mazzola et al., 2007). In contrast, the same

mutant displayed a reduced ability to control the infection by *P. infestans* in tomato, in comparison to the wild-type SS101, indicating that the antibiotic was active in the control of this other pathosystem (Tran et al., 2007). These results demonstrate that mechanisms of biological control with a given PGPR strain can be specific to particular pathosystems.

3.1.3. Antibiotic production and biocontrol in bacilli PGPR

For bacilli-PGPR, although antibiotic production is the most widely studied mechanism, much remains to be done in order to ascertain its role *in situ* (Emmert and Handelsman, 1999; Ongena and Jacques, 2008; Shoda, 2000). To present the current overview concisely, some of the most representative studies linking antibiotic production by bacilli-PGPR strains and disease control are summarized in Table 1.3. This table shows the specific bacterial strain, pathogen, experimental approach, and test condition that were used in each study, considering that all those factors are critical to determine the practical implications. Those studies have successfully proven the inhibitory properties of the listed antibiotics against specific fungal and bacterial plant pathogens. However, the role for production *in situ* of those antibiotics by bacilli-PGPR strains and its agronomic significance are less clear. Only the studies conducted by Silo-Suh et al. (1994), with kanosamine and zwittermicin, and by Asaka and Shoda (1996), with Iturin A and surfactin, have included the screening *in planta* of knock-out mutants. Therefore, the most conclusive evidence for the significance of antibiotic production *in situ* is available for the strains *B. cereus* UW85 and *B. subtilis* RB14. This strongly suggests that this mechanism has agronomic relevance for these two strains, but in the case of UW85, this aspect would be limited by the

existing restrictions to the use of strains belonging to *B. cereus*, which can be a food contaminant.

Research on the role for bacilli-produced antibiotics in biocontrol has involved a large number of strains. However, the most in-depth studies have been conducted on three specific strains: *B. cereus* UW85, *B. subtilis* RB14, and *B. amyloliquefaciens* FZB42. For *B. cereus* UW85, studies conducted by Silo-Suh et al. (1994) demonstrated the role of zwittermicin A and an unknown antibiotic, later identified as kanosamine (Milner et al., 1996a), in biocontrol of *Phytophthora medicaginis* damping-off in alfalfa. Using the purified compounds, they observed that both antibiotics prevented the disease causing different effects on the pathogen's germ tubes: zwittermicin A reduced its elongation and kanosamine caused its swelling. The linkage between production of these antibiotics and biocontrol started with the screening of a library of 2,682 mutants generated with Tn*917* or mitomycin C treatment. Five of those mutants were substantially reduced in antibiotic accumulation and disease-suppressive activity. Of all the mutants in the library, 1,700 were screened for disease-suppressive activity, revealing that three mutants with reduced activity also accumulated less of both antibiotics. Additionally, the amount of antibiotic accumulated by the mutants was significantly correlated with the level of disease suppression. Additional evidence came from the use of cultures of the mutant UW030, which was deficient in production of both antibiotics. In this experiment, only 9% of the plants survived in infested vermiculite inoculated with UW30, while cultures of UW85 (wild-type) resulted in 76%. Moreover, this protection could be restored by addition of the purified compounds, reaching 98 and 35% of plant survival with the highest concentrations of zwittermicin A and

kanosamine, respectively. Besides these initial studies, genetic analysis of zwittermicin A production was achieved generating a library of 4,800 Tn*5401* mutants (Emmert et al., 2004). Nine of those mutants did not produce detectable amounts of the antibiotic, whereas one mutant produced eightfold more than the parent strain. Sequence analysis of the DNA flanking the transposon insertions in six of the nine non-producing mutants revealed the presence of different genes involved in peptide and polyketide biosynthesis, as well as some enzyme homologs. These results suggest that zwittermicin A is synthesized by a mixed nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathway (Emmert et al., 2004).

In the case of *B. subtilis* RB14, research has focused on production of the antifungal lipopeptide iturin A and, to a lesser extent, of surfactin (Asaka and Shoda, 1996). This strain had previously shown the capacity to produce both antibiotics and inhibit some fungi *in vitro*. Therefore, studies were conducted to test the potential of RB14 to suppress damping-off of tomato seedlings caused by *Rhizoctonia solani* in pot experiments. Treatment of infested soil with culture broth, cell suspension, and centrifuged culture broth of this strain reduced disease incidence at least 60% and increased t length and weight of shoots by over 500% and 650%, respectively. These results were accompanied by detection of iturin A and surfactin in soil after treatment, suggesting a role for these two antibiotics in disease reduction. This hypothesis was tested comparing the performance of the mutant RΔ1, derived from RB14 by disruption of the gene *lpa-14*, which is required for the production of both antibiotics, to the wild-type strain and a restored transformant carrying the intact gene in the plasmid pC115. Results showed that the knock-out mutant RΔ1 was significantly less effective in controlling the disease than the wild type strain and the restored transformant. In addition, no statistical difference was found between

the pots treated with the RΔ1 mutant and those inoculated only with the pathogen or between the pots treated with the wild-type strain and those with the restored transformant. All these results strongly supported the involvement of iturin A and surfactin in the control of *R. solani*-damping off in tomato seedlings and encouraged further investigations. One of these further studies aimed to identify, clone, and sequence the iturin A synthetase operon (Tsuge et al., 2001). It was established that the iturin A operon spans a region that is more than 38 kb long and is composed of four open-reading frames: *ituD*, *ituA*, *ituB*, and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase and its disruption results in deficient production of the antibiotic. The second gene, *ituA*, encodes a 449-kDa protein with three functional modules homologous to fatty acid synthetase, amino acid transferase, and peptide synthetase. The third and the fourth genes, *ituB* and *ituC*, encode for 609- and 297-kDa peptide synthetases that harbor four and two amino acid modules in each case. Promoter analysis of *ituA*, which was achieved by primer extension, revealed low levels of homology of the -10 and -35 consensus sequences of σ^A (Haldenwang, 1995). However, this promoter and the flanking sequences of the operon showed high homology with those of the *xynD* gene of *B. subtilis* 168, suggesting that horizontal transfer might have occurred. Interestingly, Tsuge et al. (2001) also found that mycosubtilin, an antibiotic that belongs to the same family (see Table 1.2), possesses almost the same structure as iturin A and that the genes *ituD, ituA,* and *ituB* share high levels of homology with the counterpart genes *fenF* (79%), *mycA* (79%), and *mycB* (79%), respectively.

The third well-studied strain is *B. amyloliquefaciens* FZB42, which is the only bacilli-PGPR strain whose whole genome has been sequenced (Chen et al., 2007a). With a size of 3,918-kb, the FZB42 genome contains an estimated 3,693 protein-coding sequences and 8.5 % of it is

devoted to antibiotic production. The majority of these protein-coding sequences was found to be conserved in the closely related *B. subtilis* and *B. licheniformis*. However, 214 genes were unique to FZB42, many of which are clustered in 17 DNA islands, and some of which appear to be linked with plant-associated lifestyle. For example, out of six gene clusters total, two clusters encoding for polyketides synthases (*pks2* and *pks3*) and one for a nonribosomal peptide synthase (*bmy*) are unique insertions in the FZB42 genome and are not present in the model strain *B. subtilis* 168 (a non-typically recognized PGPR strain) (Koumoutsi et al., 2004). Other important difference between the genomes of FZB42 and 168 relate to the production of a polyketide (presumably bacillaene), surfactin, and fengycin. Although both strains posses the operons needed for production of these antibiotics, only FZB42 can produce them due to a frameshift mutation in the *sfp* gene of 168, which specifies an inactive protein required for activation of the respective antibiotic synthetases (Stein, 2005).

FZB42 contains the operons *srf*, *fen*, and *bmy*, which are responsible for the synthesis of three lipopeptides with antifungal activity: surfactin, fengycin, and bacillomycin D, respectively (Koumoutsi et al., 2004). The fengycin (*fen*) and surfactin (*srf*) operons are organized and located as in *B. subtilis* 168, whereas the *bmy* island is inserted about 25 kb distant from the *fen* operon. The 37.2-kb *bmy* gene cluster is inserted at the same position as iturin A gene cluster in *B. subtilis* RB14 and shows a similar genetic organization following the colinearity rule. Like in the iturin A operon of RB14, the first open-reading frame of the FZB42 bacillomycin D operon, *bmyD*, encodes a putative malonyl coenzyme A transacylase. Similarly, the other three open-reading frames encode for the rest of the enzymes in the operon: BmyA (3982 aminoacids), BmyB (5633 amino acids), and BmyC (2619 amino acids). Production of bacillomycin D in *B.*

amyloliquefaciens FZB42 is regulated in multiple layers (Koumoutsi et al., 2007). Using 5' deletion analysis of the *bmyD* promoter region and primer extension, it was found that a single σ^A promoter is responsible for the expression of the operons. This promoter involves upstream DNA *cis*-acting elements and is favored by the small regulatory protein DegQ. In addition, the global regulators DegU and ComA are required for the full transcriptional activation of *bmy.* Both DegU and a transmembrane protein of unknown function, denominated YczE, regulate the later level of gene expression through postranscriptional effects. To determine the role for these lipopeptides in diseases suppression, mutant analysis was performed using cassette mutagenesis (Koumoutsi et al., 2004). Mutants lacking production of single lipopeptides were found to retain *in vitro* inhibition of *Fusarium oxysporum* f.sp. *cucumerinum*. However, a double mutant lacking both bacillomycin D and fengycin demonstrated greatly reduced antagonistic activity, suggesting that the antibiotics act synergistically. Nevertheless, further investigations with FZB42 involving actual pathosystems are still required, as no report of *in planta* tests showing equivalent results was found among the literature reviewed for this manuscript. Such tests are necessary to determine if these lipopeptides are causally linked to biocontrol and if their production is relevant for the use of FZB42 in agriculture.

Additionally, *B. amyloliquefaciens* FZB42 possesses three other gene clusters involved in the synthesis of the polyketides bacillaene (*pks1*), macrolactin (*pks2*), and difficidin/oxydifficidin (*pks3*), all of which exhibit antibacterial activity (Chen et al., 2006; Schneider et al., 2007). FZB42 polyketide production is associated with three very large modular PKS systems of the *trans*-acyltransferase type, covering 196.34-kb total (Chen et al., 2006). *pks1* is an ortholog of the *pksX* operon of the model strain *B subtilis* 168, whereas the *pks2* and *pks3* clusters are

unique. The role of *pks1* and *pks3* in production of bacillaene and difficidin, respectively, and their contribution for *in vitro* inhibition of the phytopathogenic bacterium *Pectobacterium carotovora* were confirmed using both single and double mutants created by cassette mutagenesis (Chen et al., 2006). On the other hand, the *pks2* cluster was subsequently found to be responsible for the biosynthesis of the antibiotic macrolactin (Schneider et al., 2007). This compound displays a weaker activity than the other two polyketides, and its role in plant pathogenic bacteria suppression is less clear. The significance of polyketide production by FZB42 *in planta* it is not known yet. Production of the polyketide difficidin and the dipeptide bacilysin was shown to be needed for the control of fire blight in apple blossoms, caused by *Erwinia amylovora*, when culture broths of FZB42 and deficient mutants were sprayed (Chen et al., 2009b). This clearly indicates that difficidin and bacilysin produced by FZB-43 are active *in planta*. Nonetheless, it is not clear yet if the level of production of these compounds *in situ* is high enough to be effective when vegetative cells or spores are applied.

3.2. Lytic Enzymes

Support for the contribution of lytic enzymes in biocontrol exerted by PGPR is not clearly established (Banerjee et al., 2006). Some studies are contradictory as the evidence suggests a role in some cases but not in others (De Boer et al., 1998). However, production of lytic enzymes has traditionally been considered to be a mechanism of biological control (Compant et al., 2005; Siddiqui, 2006). The rationale behind this consideration is that chitinases, β-glucanases, celluloses, and proteases can degrade cell walls of plant pathogenic fungi and oomycetes (Banerjee et al., 2006). The consideration of lytic enzyme production as a mechanism of

biocontrol by PGPR is mainly based on indirect evidence such as: 1) detection of lytic enzyme production in bacterial strains with antagonistic properties (De Boer et al., 1998; Kishore et al., 2005; Singh et al., 1999); 2) *in vitro* cell-wall degradation or inhibition of hyphal elongation and propagule germination by bacterial crude extracts or purified enzymes (Huang and Chen, 2004; Singh et al., 1999); and 3) increases of indigenous lytic populations by application of specific substrates that result in disease reduction (Hallmann et al., 1999).

To date, very few studies provide evidence for a causal link between lytic enzyme production and biocontrol by PGPR using mutant analysis. One of these studies compared the strain *Enterobacter agglomerans* IC1270, which produces a chitinolytic enzyme complex, to Tn*5* mutants for controlling damping-off in cotton, caused by *R. solani* (Chernin et al., 1995). The mutants IC1270-E1 and IC1270-2h, both deficient in chitinolytic activity, were unable to control the disease, suggesting a role for chitinase production. However, the mutant IC1270-E1, which had a single Tn*5* insertion, was also deficient in antibiotic production and the mutant IC1270-2h showed two transposon insertions. Taking all these results into account, the inability of the mutants to control *R. solani*-damping-off in cotton should be interpreted carefully as activities other than chitinase production could have been affected in the mutants. Further characterization showed that a transformant of *Escherichia coli* JM109 carrying the *E. agglomerans* C1270-chitinase gene inhibited *R. solani* on plates and reduced damping-off of cotton under greenhouse conditions (Chernin et al., 1997). This finding supported the role for this enzyme in biological control of damping-off in cotton, but the role for chitinase production by the wild-type strain still requires clarification.

Another study involving mutant analysis to investigate the causal link between lytic enzyme production and biocontrol in PGPR was conducted with the strain *Lysobacter anzymogenes* C3, a β-1,3-glucanase producer (Palumbo et al., 2005). In this case, a mutant of *L. anzymogenes* C3 was generated by simultaneous disruption of the three genes responsible for β-1,3-glucanase production in the wild-type strain. Although this mutant retained antimicrobial activity *in vitro*, it showed significantly reduced control of damping-off of sugar beet, caused by *Pythium ultimum* var. *ultimum*, and Bipolaris leaf spot of tall fescue, caused by *Bipolaris sorokiniana*. These results clearly suggested that β-1,3-glucanases produced by *L. anzymogenes* C3 were involved in the biocontrol activity exerted by this strain.

In the particular case of bacilli-PGPR, direct evidence for lytic enzyme production as a cause of biocontrol is even scarcer. To our knowledge, no study has yet compared the biocontrol performance of a chitinase- or glucanase-producing bacilli strain to a non-producing mutant. The evidence available is of an indirect nature including reports that antifungal-bacilli strains displaying chitinolytic (Cazorla et al., 2007; Huang et al., 2005a; Jung et al., 2003; Singh et al., 1999) and glucanolytic activities (Aono et al., 1995). These bacilli-lytic enzymes have been shown to affect hyphae or propagules of pathogenic fungi *in vitro*, suggesting a role for these enzymes in the antifungal activity displayed by bacterial cells (Aono et al., 1995; Huang and Chen, 2004; Huang et al., 2005a). However, some studies provide contradictory evidence and indicate that the activity of lytic enzymes should not be used as a reliable criterion for antifungal activity in bacilli as no clear correlation between those two parameters is seen in this group of bacteria (Aktuganov et al., 2003).

Some bacilli-produced enzymes other than those responsible for cell-wall degradation have more recently been suggested as possible contributors for biological control. One of these cases is the production of *N*-acyl homoserine (AH) lactonases, which degrade key molecules involved in communication of phytopathogenic bacteria in a process known as *quorum quenching* (Dong et al., 2002). The role of this group of enzymes in *Bacillus* spp. is not clear. However, the suggestion of a possible participation in biocontrol activity comes from experiments with a transformant strain of the bacterial pathogen *Erwinia carotovora* with a gene for a AH-lactonase from *Bacillus* sp. 240B1. In this study, the transformant showed reduced release of signal molecules, lower pectolytic enzyme activity, and attenuated pathogenicity on several cultivated plants (Dong et al., 2000). Similarly, production of extracellular proteases by bacilli strains has been associated with nematode control via cuticle degradation (Lian et al., 2007; Niu et al., 2007). The effectiveness of these bacilli-produced proteases was proven using extracts from bacterial cultures, which degraded the cuticle of the free-living nematode *Panagrellus redivius*. In contrast, extracts from a knock-out mutant defective in protease production displayed a significantly reduced nematode-degrading activity (Niu et al., 2007). Further research is needed to establish the role of production of these enzymes by AEFB strains *in situ* and the implications for practical use of bacilli-PGPR.

3.3. Siderophores

Siderophores are defined as low molecular weight Fe^{+3}-transport agents with a very high affinity, whose function is to supply iron to the cell (Leong, 1986). Production of these compounds has traditionally been considered as a PGPR mechanism of biocontrol by depriving

pathogens of iron (Kloepper et al., 1980). This aspect has been intensively studied regarding both plant growth and disease control (Neilands and Leong, 1986), but mostly in relation to fluorescent *Pseudomonas* spp. (Leong, 1986). A review focused on biocontrol by this bacterial group (Haas and Défago, 2005) reflects the complexity of this trait, mentioning that siderophores seem to be relevant for biocontrol in some, but not all, plant-pathogen systems. Siderophore-mediated deprivation seems to be a contingent mechanism because it works much better at pH 8 than at pH 6 due to Fe^{+3} species increase solubility with decreasing pH. In addition, research aimed at understanding the environmental factors modulating siderophore biosynthesis by *Pseudomonas fluorescens* established that this mechanism was regulated by diverse minerals and carbon sources (Duffy and Défago, 1999).

In contrast to pseudomonads, very few siderophores have been reported in bacilli, and no clear linkage between them and biological control has been established. Studies involving bacilli-produced siderophores have not typically included agricultural applications and many have only focused on basic chemical or molecular biology aspects (Grossman et al., 1993). One of the most studied bacilli siderophores is schizokinen, which is comprised of a residue of citric acid and produced by *Bacillus megaterium* ATCC19213 and various strains of cyanobacteria (Neilands and Leong, 1986). This siderophore seems to be produced under natural soil conditions as suggested by its presence in soil of rice fields after the annual flooding period (Akers, 1983). Some studies have indicated that, besides uptake of iron, aluminum can also be mobilized by schizokinen and its related siderophore *N*-deoxyschizokinen, both produced ATCC19213 (Hu and Boyer, 1996). This constitutes an interesting finding taking into account that aluminum and iron are unavailable under similar soil conditions (Marschner, 1995). Another

Bacillus-produced siderophore is bacillibactin, also classified as an antibiotic (Table 1.2). This siderophore has been reported to be produced by different strains of *B. subtilis*, where some genetic details related to its synthesis have been described (Miethke et al., 2006). Bacillibactin was also recently found to be expressed in the PGPR strain *B. amyloliquefasciens* FZB42 (Chen et al., 2007a), but its role in plant-bacterium interactions has not yet been investigated.

3.4. Induced Systemic Resistance

Induced systemic resistance (ISR) is defined as a state of enhanced defensive capacity developed by a plant when stimulated by rhizobacteria (van Loon et al., 1998). The first reports on ISR were made in 1991 (van Peer et al., 1991; Wei et al., 1991) and, since then, much research has been conducted in this field. Different from the rest of the potential biocontrol mechanisms, use of defined mutants is not required to definitively prove that ISR is occurring. The evaluation *in planta* with physical separation between the rhizobacterium (inducing agent) and the pathogen is considered to be strong enough proof (van Loon et al., 1998). If the resulting plant response is a reduction in the level of disease, this reduction is interpreted as being necessarily mediated by the plant and rules out any bacterium's direct effect on the pathogen. This is the strategy traditionally followed by researchers, including the two first reports mentioned above. One of these studies was conducted with *Fusarium oxysporum* f.sp. *dianthi*, the causal agent of Fusarium wilt of carnation, which was kept separate from the strain *Pseudomonas* sp. WCS417r, the ISR-inducing agent, using a split-root system (van Peer et al., 1991). Similarly, the other study also ensured this physical separation, but in this case using a

different experimental model: root-colonizing bacteria as inducing agents and a foliar disease, the anthracnose of cucumber caused by *Colletotrichum orbicularae* (Wei et al., 1991).

After the initial reports of ISR induced by pseudomonads, a large number of studies were published using bacilli as elicitors of ISR. Kloepper et al. (2004) summarize the bacilli strains and pathosystems for which ISR is reported, as well as the physiological and molecular changes that are known, so far, to take place in the bacilli-induced plant. A very relevant study was developed by Yan et al. (2002), in which results allowed comparison at the induction pathways between pseudomonads and bacilli. This work tested the strains *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, which previously had shown to induce systemic resistance, on three tomato lines: salicylic acid (SA)-hydrolaxylase transgenic tomato (*nahG*), ethylene insensitive mutants (*Nr/Nr*), and jasmonic acid insensitive mutants (*def1*). Interestingly, SE34 and 89B61 just elicited ISR in the first line of plants, suggesting that similar to pseudomonads, plant response to bacilli can be SA-independent but ethylene- and jasmonic acid- dependent.

This has been the pattern constantly found in *Pseudomonas* spp. strains and has become a paradigm in pseudomonads-elicited ISR-signaling research (Van Loon and Bakker, 2003). Nevertheless, results obtained by (Ryu et al., 2003b) suggest that ISR elicitation by bacilli can actually follow different transduction pathways depending on the strain and plant-pathogen system. Although in most cases results were in agreement with the typical pathway in response to *Pseudomonas* spp. inoculation, *B. pumilus* strain SE34 elicited ISR in *nahG* plants against *Pseudomonas syringae* pv. *maculicola* but did not against the other pathovar, *P. syringae* pv. *tomato*, suggesting that, for the latter pathovar, ISR triggered by SES34 is SA-dependent.

Another contrasting result found in this study was that ISR elicited by *Bacillus pumilus* T4 can sometimes be NPR1- and jasmonic acid- independent, compounds that are required in plant response to the rest of bacilli and pseudomonads PGPR.

In comparison with pseudomonads, ascertaining the bacterial determinants of bacilli-elicited ISR has been less investigated. Two relevant studies can be cited in this regard, where production of volatile organic compounds (VOCs) (Ryu et al., 2004) and antibiotics (Ongena et al., 2007) seem to be involved in bacilli's ability to trigger ISR. In the first mentioned research, *Arabidopsis* seedlings were exposed to bacterial volatile blends from the well-known PGPR strains *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, using divided petri dishes to keep seedlings physically separated from PGPR, and inoculated with the bacterial pathogen *Erwinia carotovora* subsp. *carotovora*. Disease severity was significantly reduced compared with seedlings not exposed to the bacterial volatiles before pathogen inoculation. Interestingly, it was found that just 4 days of exposure to VOCs from rhizobacteria was sufficient to activate ISR in the seedlings, and that the volatile emissions included low-molecular weight hydrocarbons such as 2,3-butanediol. Exogenous application of this compound showed to be capable of inducing resistance in this plant-pathogen system, and to be dependent on ethylene and independent of SA and jasmonic acid. Further research is still required in order to establish whether these VOCs can actually operate by eliciting ISR under natural conditions where bacilli are inoculated.

On the other hand, surfactin and fengycin, two lipopeptide antibiotics (see Table 1.2), were more recently suggested to trigger ISR (Ongena et al., 2007). For this purpose, the model non-PGPR strain *B. subtilis* 168 and three mutants of it were used. As mentioned above, *B. subtilis*

168 does not produce lipopeptides as a consequence of a dysfunctional *sfp* gene. For this reason, a mutant *B. subtilis* 2500 was created by inserting a functional *sfp* gene from another strain, *B. subtilis* S499, into the chromosome of 168. This mutant was able to produce high levels of surfactin but still very low amounts of fengycin. Because of this, another mutant, designated 2508, was obtained by replacement of the weak fengycin promoter (*pfen*) by the strong *amyQ* promoter, effectively resulting in a high producer phenotype for both antibiotics. The third mutant used in this research, 2504, was a derivative of 2508 that also secretes fengycins in large amounts but is impaired in surfactin production. Comparison of the capacity of the wild-type strain 168 and its three mutants 2550, 2508, and 2504 to reduce disease incidence of *Botrytis cinerea* on leaves of both bean and tomato showed an effect of the antibiotic-producing phenotypes to elicit ISR. While plants inoculated with 168 (non-producer) displayed a similar disease incidence to the non-inoculated control, inoculation with the three antibiotic-producing mutants significantly reduced it, especially 2508, which produces high amounts of both fengycin and surfactin. This research was conducted using soil, and therefore, the mechanism could operate in natural environments. However, some further research would be useful in order to determine whether specific bacilli-PGPR strains, which are natural antibiotic producers, secrete a sufficient amount of these compounds to stimulate the plant defenses.

4. Direct Mechanisms

Although direct plant growth promotion by bacilli is widely documented (Banerjee et al., 2006; Kloepper et al., 2004; Vessey, 2003), the underlying mechanisms remain poorly understood. This is more evident when surveying the literature, where it is possible to see that

most of the mechanisms commonly attributed to bacilli-PGPR are based on indirect evidence and extrapolation of findings made with azospirilla and pseudomonads (Table 1.4). Thus, the evidence supporting specific plant growth promoting mechanisms of bacilli is commonly based on 1) determination of the presence of bacterial traits usually referred to as having beneficial effects on plants (Asghar et al., 2002; Joo et al., 2004); 2) application of crude bacterial extracts or purified bacterially-produced compounds that promote plant growth (Krebs et al., 1998; Pilet and Saugy, 1987); and 3) detection of changes in physiology or mineral content in plant tissue (Joo et al., 2005; Timmusk and Wagner, 1999; Toro et al., 1997).

These approaches have been extremely useful as practical methods for selective isolation and screening of PGPR, and sometimes high correlations between the presence of those traits and plant growth promotion are found. However, they are not enough to prove that those mechanisms are being expressed *in situ* and at a sufficient level to impact plant growth. This is reflected in several studies where contradictory results are found, showing that not all the strains displaying a certain *in vitro* test necessarily promotes plant growth, or that this can be site-dependant (Cattelan et al., 1999). For this reason, these studies should be considered as valuable preliminary information if practical implications are the goal, but further research should be conducted in order to uncover key points for PGPR performance optimization. However, design of experimental approaches to overcome these limitations is not an easy task to accomplish, as many environmental factors impact the final result, affecting not only the PGPR-strain involved, in terms of colonization (Maplestone and Campbell, 1989) and physiology (Vilain et al., 2006), but also the plant, which can be responsive or not depending on the specific mechanism and the environment (Kloepper et al., 2007).

Table 1.4 Experimental evidence for direct mechanisms of plant growth promotion by bacilli strains

Mechanism	Strain	Plant	Experimental Approach	Test Condition	Reference
Nitrogen fixation	*Paenibacillus polymyxa* ATCC10343 *Bacillus* sp. SVPR30	Rice	*In vitro* biochemical test	Greenhouse / vermiculite	Beneduzi et al. (2008b)
	Paenibacillus polymyxa RC05	Sugar beet	*In vitro* biochemical test	Greenhouse and field	Çakmakçi et al. (2006)
	Paenibacillus sp. SBR5 *Paenibacillus* sp. CSR16 *Paenibacillus* sp. EsR7	Wheat	*In vitro* biochemical test	Greenhouse / vermiculite	Beneduzi et al. (2008a)
Phosphorus Solubilization					
Inorganic phosphate	*Bacillus firmus* NCIM-2636	Rice	- *In vitro* biochemical test - Plant mineral content	Field	Datta et al. (1982)
	Bacillus subtilis[a]	Onion	- *In vitro* biochemical test - Plant mineral content	Greenhouse / soil In presence of mycorrhiza	Toro et al. (1997)
Organic phosphate (Phytate)	*Bacillus mucilaginosus* NKTS-3	Tobacco	Transgenic strain expressing the *Aspergillus fumigatus* phytase gene	Greenhouse / soil	Li et al. (2007)
	Bacillus amyloliquefaciens FZB45	Maize	- *In vitro* biochemical test - Knock-out mutant analysis	Growth chamber / Nutrient solution	Idriss et al. (2002)
Potassium Solubilization	*Bacillus edaphicus* NBT	Wheat	- *In vitro* biochemical test - Mutants created by UV	Greenhouse / soil	Sheng and He (2006)
IAA production	*Bacillus polymyxa* L6 *Bacillus polymyxa* L5 *Bacillus megaterium* S49 *Bacillus megaterium* S72 *Bacillus brevis* S72	Beans	- *In vitro* biochemical test - Evaluation of nodulation by rhizobia	Growth chamber / Potting mix	Srinivasan et al. (1996)
	Bacillus subtilis FZB14	Wheat	- *In vitro* biochemical detection - Specific fractions of culture filtrates	*In vitro* test on wheat coleoptile	Krebs et al. (1998)
	Bacillus amyloliquefaciens FZB42	*Lemna minor* Aquatic plant	- Detection in culture filtrates - Tryptophan auxotrophic mutants - Knock-out mutants	48-well microtiter plates / Nutrient solution	Idris et al. (2007)
Gibberellin production	*Bacillus pumilus* CECT 5105 *Bacillus licheniformis* CECT 5106	Alder	- *In vitro* biochemical detection - Culture filtrates addition	Growth chamber / Vermiculite	Gutiérrez-Mañero et al. (2001)
	Bacillus cereus MJ-1	Red pepper	- *In vitro* biochemical detection - Gibberellin content of plant tissue	Growth chamber / soil	Joo et al. (2005)
	Bacillus cereus MJ-1 *Bacillus macroides* CJ-29 *Bacillus pumilus* CJ-69	Red pepper	- *In vitro* biochemical detection - Gibberellin content of plant tissue	Growth chamber / soil	Joo et al. (2004)
Cytokynin production	*Paenibacillus polymyxa* B2	Arabidopsis	*In vitro* biochemical detection	*In vitro* plant test	Timmusk et al. (1999) Timmusk and Wagner (1999) Timmusk (2003)
	Bacillus amyloliquefaciens FZB24	Radish	- *In vitro* biochemical detection - Specific fractions of culture filtrates	*in vitro* test on cotyledons	Krebs et al. (1998)
ACC-deaminase production	*Bacillus circulans* DIC1 *Bacillus firmus* DUC2 *Bacillus globisporus* DUC3	Canola	*In vitro* biochemical test	Greenhouse / soil	Ghosh et al. (2003)
Volatiles	*Bacillus amyloliquefaciens* IN937a *Bacillus subtilis* GB03 *Bacillus subtilis* 168	Arabidopsis	- Detection of bacterially –produced compounds - Knock-out mutants of 168	*In vitro* plant test	Ryu et al. (2003a)

[a]Strain not defined in the reference.

To date, the use of mutants to investigate direct mechanisms of growth promotion by bacilli-PGPR has not been used as frequently as for the investigation of the role of antibiotics ; however four such studies have been published (Idris et al., 2007; Idriss et al., 2002; Ryu et al., 2003a; Sheng and He, 2006).

In the next part, the two most studied groups of direct mechanisms are discussed: mineral solubilization and phytohormone production. Most of the literature is focused on these two mechanisms, which has probably been encouraged in part by the previous knowledge about the impact of mineral nutrients and hormones in plant production. Nevertheless, there are other potential mechanisms much less investigated so far, like the effect of certain bacilli strains on soil structure. For example, strain *P. polymyxa* CF43 has consistently been found to produce levan, a fructosyl polymer responsible for increased soil mass adhering to roots, when this strain is inoculated (Bezzate et al., 2000; Bezzate et al., 1994). As efforts continue to use PGPR for improving soil structure, additional strains of PGPR should be examined for this trait.

4.1. Mineral solubilization

Increased nutrient availability has been reported as a mechanism by which PGPR can benefit plants (Banerjee et al., 2006). The term *biofertilizers* has been used to describe soil microorganisms that increase the availability and uptake of mineral nutrients for plants (Vessey, 2003). Most work on the role of PGPR as biofertilizers has involved nitrogen (N) fixation and, by far, *Azospirillum* is the most relevant group in this regard (Bashan et al., 2004). Some bacilli

PGPR strains also fix N (Grau and Wilson, 1962; Zlotnikov et al., 2001) and solubilize potassium (Sheng and He, 2006). However, the impact of bacilli-PGPR on plant nutrition has mainly been focused on phosphorus (P) (Rodriguez and Fraga, 1999).

Although it has been known for many years that some bacilli strains are capable to fix N (Grau and Wilson, 1962), the role of this trait in bacilli for plant growth promotion is greatly unclear. Evidence supporting N fixation as a mechanism of beneficial bacilli is mainly from reports that some PGPR strains grow on minimal media without any N source (Park et al., 2005) and produce nitrogenase (Çakmakçi et al., 2006). Nevertheless, some N-fixing bacilli have been shown to improve plant growth under N-limited conditions (Beneduzi et al., 2008a; Beneduzi et al., 2008b; Çakmakçi et al., 2006). For instance, Beneduzi et al. (2008b) found a strong growth promotion effect of *Bacillus* sp. SVPR30 on rice, 15 and 30 days after sprouting. This strain was selected because of its considerably high N fixation capacity, but it also produces high levels of indole-acetic acid (IAA), produces siderophores and solubilizes phosphate, making unclear the actual role of each specific mechanism. A similar situation was reported by Beneduzi et al. (2008b) who observed wheat growth promotion by three different strains (see Table 1.4) of *Paenibacillus*, but all of them expressing other *in vitro* putative PGPR-traits simultaneously. Some ecological studies have been conducted on these N-fixing bacilli. On the other hand, regarding solubilization of potassium, Sheng and He (2006) reported that wild-type *Bacillus edaphicus* NBT, a potassium-solubilizing strain, and a mutant improved in this capacity promoted plant growth and K uptake. This effect was reduced in mutants showing reduced and no solubilization, suggesting that solubilization of potassium could be the mechanism involved. However, these mutants were created by UV treatment and, then, not defined. Therefore, these

mutants could have been impaired in other mechanisms such as IAA production or increased plant N and P uptake, which were seen in the wild-type strain.

A special case of nutritional improvement of plants by bacilli inoculation is the enhancement of legume nodulation. This phenomenon was initially described in soybean grown under growth chamber and field conditions (Halverson and Handelsman, 1991). This research effectively demonstrated that seed treatment with *B. cereus* UW85 increased nodulation of soybeans in three field seasons (31 to 133%) and in three different sterilized soils in the growth chamber (34-61%). These results suggest that the phenomenon was not dependent on the soil flora. Srinivasan et al. (1996) reported that 5 IAA-producing *Bacillus* spp. isolates promoted root growth and nodulation when inoculated with *Rhizobium etli* TAL182 on beans. However, these benefits could not be explained solely by production of IAA because mutants lacking IAA production reduced nodulation, suggesting that factors in addition to IAA might contribute to promotion of nodulation by the wild-type strain. A more recent study of enhanced nodulation by bacilli (Estevez de Jensen et al., 2002) showed that inoculation of bean with *B. subtilis* GB03 and *B. subtilis* MBI600 increased yield and reduced *Fusarium* root rot under field conditions. Although these effects were not always statistically significant, GB03 was effective during two seasons, and MBI600 was the only efficient treatment during the last season of evaluation. It is important to keep in mind that MBI600 is commercialized (see Table 1.1) not only as a biological fungicide but also as a helper for increasing nodulation by N-fixing bacteria in several legumes (www.beckerunderwood.com/en/products/subtilex).

As mentioned above, biofertilization using bacilli-PGPR has been commonly seen as an alternative in plant P nutrition. One of the reasons for this is the importance of P fertilization, as this element is required for every aspect of cell biology from energy metabolism to the structure of genetic material, making P the world's second most applied fertilizer after N (Goldstein and Krishnaraj, 2007). This need for P fertilization is even higher taking into account that the element is often present in soil as unavailable forms, such as insoluble complexes with calcium (in high soil pH) and free oxides and hydroxides of aluminum and iron (in low soil pH). Also, it is part of large organic molecules that cannot be taken up by the plant (Rodriguez and Fraga, 1999). Likewise, P can also be fixed to the surface of clays and organic matter, making it less available for the plant (Fox and Kamprath, 1970a). With this in mind, and driven by the current search for cleaner alternatives for agriculture, use of microbial P solubilizers has been seen as a promising option for improving plant P nutrition (Goldstein and Krishnaraj, 2007; Gyaneshwar et al., 2002; Richardson, 2001). In the specific case of PGPR, many aspects are still unclear on P solubilization; nonetheless, important insight has been gained on applied (Rodriguez and Fraga, 1999) and molecular aspects (Rodríguez et al., 2006).

As in the rest of the mechanisms mentioned in this manuscript, P solubilization as a mechanism of plant growth promotion by bacilli-PGPR is partially based on research conducted with other bacterial groups. Hence, the actual significance of this mechanism in the bacilli-plant-soil interaction is not sufficiently clear. *In vitro* solubilization of P by bacilli, from both organic and inorganic sources, has been widely documented (Richardson, 2001; Rodriguez and Fraga, 1999; Vazquez et al., 2000), but the impact of P solubilization on P nutrition of plants is contradictory. For instance, Toro et al. (1997) showed that inoculation with a strain of *B. subtilis*,

which solubilized P *in vitro*, significantly increased shoot dry weight and P content of onion in a low-P soil, regardless of the presence of mycorrhiza. When rock phosphate was added, the inoculation was only effective in the presence of the fungus. This suggested that bacterial P-solubilization was exerted in soil helping to promote plant growth. In contrast, de Freitas et al. (1997) tested several isolates of *Bacillus* spp. on canola, which had been selected for their ability to solubilize phosphate and produce IAA *in vitro*. Even though some isolates significantly increased plant height, none of those inoculants promoted total P uptake. This would suggest that the capacity of these strains to solubilize P from insoluble calcium phosphate and rock phosphate *in vitro* was not exerted in soil.

In relation to the effect of bacilli P solubilization from organic sources on plant growth promotion, scarce but valuable articles have been published. This research has basically involved just one type of P-containing organic molecule, phytic acid[4]. This compound accounts for 20 to 50% of soil organic P, which may represent up to 50 to 80% of total (inorganic+organic) soil P (Richardson, 2001). Phytases are enzymes that hydrolyze phosphomonoester bonds from phytic acid, thereby releasing inorganic orthophosphates (Mullaney and Ullah, 2003) which are the main P forms absorbed by plants (Marschner, 1995). There are at least three different classes of phytases, which can be produced by diverse groups of organisms such as plants, fungi, and bacteria (Mullaney and Ullah, 2003). Inoculation of phytase-producing microorganisms has commonly been justified by the low ability of plants to secrete their own phytases to hydrolyze phytic acid (Hayes et al., 2000a; Richardson et al., 2001a). In the specific case of phytase-producing bacilli, Idriss et al. (2002) evaluated the role of extracellular phytase activity of *B.*

[4] Also referred to in literature as phytate, *myo*-inositol hexakis dihydrogen phosphate, *myo*-inositol phosphate, or phytin (when complexed with Ca^{+2} or Mg^{+2}).

amyloliquefaciens FZB45 in plant growth promotion. A previous characterization of the FZB45 phytase revealed that it was able to release 3 molecules of P_i from each molecule of phytic acid, like in other *Bacillus* phytases (Konietzny and Greiner, 2004). To evaluate the hypothesis that the capacity of some root-colonizing bacteria to make the phytate P available for plant nutrition in soil, under phosphate-starvation conditions, contributes to their plant growth-promoting activity, the performance of a phytase-negative mutant was compared to the wild-type FZB45 strain. The experiment was conducted by treating maize seedlings with culture filtrates and spores of the mutant FZB45/M2 in a soilless gnotobiotic system using nutrient solution. This mutant was derived from FZB45 by an insertional inactivation of the phytase-encoding gene. Here, inoculation with the wild-type strain and its culture filtrates significantly promoted shoot and root weight, root length, and phosphate concentration in the nutrient solution. In contrast, none of these parameters was affected by the mutant. These results suggest that phytase activity may operate as a mechanism of plant growth promotion in FZB45 and, possibly, in related strains. However, the authors clearly state that their study should only be considered as a starting point and call for verification under conditions which better mimic the natural environment.

This need for studies that mimic the natural environment was reinforced by Richardson (2001) who stated that further investigations are needed to determine the contribution of microorganisms to the mineralization of phytate in soil. Several studies report plant growth promotion by phytase-producing microorganisms other than bacilli (Richardson et al., 2001b; Unno et al., 2005) and by transgenic expression of microbial phytases in plants (George et al., 2005c; Richardson et al., 2001a). From these studies, it is clear that many external and internal factors will determine the final plant response. Among the external factors, the first to be

mentioned is that P in soil solution should be low enough for the specific plant to be responsive to P increases in the rhizosphere (Fox, 1981). Likewise, soil phytate availability and the P-fixation capacity of the particular soil should be considered. For example, positive responses to inoculation with phytase-producing microorganisms (Richardson et al., 2001b) and improved growth of phytase-expressing transgenic plants (George et al., 2005c) are only seen in soils with low P-fixation capacity, high amounts of phytate, or amendments with lime. In addition, phytase can be immobilized in soil, which limits its capacity to interact with phytate and to release P (George et al., 2005b). It is important to note that, to date, all the studies conducted on the effect of soil on phytase enzymes have used phytase from the fungus *Aspergillus niger*. This enzyme belongs to a class of phytases different from those produced by *Bacillus* spp. (Mullaney and Ullah, 2003) and both should have a distinct behavior in soil.

Another important factor to consider when assessing the role of phytase in P-nutrition is gene regulation. Makarewicz et al. (2006) provided important evidence for how gene regulation can impact phytase activity in a study with the phytase gene (*PhyC*) of *B. amyloliquefaciens* FZB45. Characterization of the upstream region revealed a σ^A-like promoter with an unusually large window of 21 bp between the -10 and -35 consensus sequences. Expression of *PhyC* was found to be dependent on the PhoP~P protein, which only increases under phosphate starvation, so that the phytase gene is expressed in these conditions. Interestingly, the promoter was expressed at levels of P concentration lower than 0.3 mM (equivalent to 9.3 mgP/L). This level was considered by the authors as ‘starvation’; however, it is much higher than the average concentration in soil (estimated to be 0.025 mgP/L) than the adequate level for most plant species (about 0.2 mgP/L) (Fox, 1981). Hence, the phytase gene of FZB-45 should be expressed

in most soils. Studies conducted by Li et al. (2007) demonstrated that inoculation with *B. mucilaginosus* NKTS-3, a transgenic phytase-expressing strain, promoted more tobacco plant growth than the wild-type strain in soil. These results suggest that the phytase gene can be expressed in soil increasing P availability. However, it is important to note that no physical and chemical properties were defined for the soil used in the study, and that the phytase gene being expressed by NKTS-3 is originally from *Aspergillus fumigatus* (Li et al., 2005) rather than a typical *Bacillus* phytase gene.

4.2. Phytohormones

The role of phytohormones in plant physiology is very well known (Weyers and Paterson, 2001), and therefore production of these substances by plant-associated microorganisms has attracted much attention . This has led to a large number of publications and the topic to be thoroughly reviewed (Arshad and Frankenberger, 1998). PGPR have been considered as a group that produces plant growth-regulating substances, and this aspect is always included in reviews addressing mechanisms for plant growth promotion by rhizobacteria (Banerjee et al., 2006; Fuentes-Ramirez and Caballero-Mellado, 2006; Vessey, 2003; Zahir et al., 2004). Specifically regarding bacilli-PGPR, the potential for production of plant growth regulators is high, as demonstrated by a large body of publications on this subject (Tsavkelova et al., 2006). However, evidence supporting the biological significance *in situ* of these substances and their contribution to plant growth promotion is scarce (Table 1.4). Most of the existing links between plant growth increases and bacilli-produced phytohormones have been based on indirect evidence following two lines: 1) the capacity of certain strains to promote plant growth and to produce

phytohormones at the same time (Asghar et al., 2002) and 2) the hormone-like plant response to addition of crude or specific fractions of culture filtrates (Krebs et al., 1998). However, no causal effect is proven in the vast majority of the publications.

No doubt the most widely studied plant hormone produced by PGPR is indole-acetic acid (IAA). In this case, there is clear evidence involving production of this compound in plant growth promotion by *Pseudomonas* spp. (Patten and Glick, 2002; Xie et al., 1996) and *Azospirillum* spp (Dobbelaere et al., 1999) PGPR, but its role for bacilli is less clear. IAA production is found in bacilli PGPR in conjunction with other traits such as production of siderophores, nitrogenase, or phosphate solubilization (Beneduzi et al., 2008a; Beneduzi et al., 2008b; de Freitas et al., 1997; Krebs et al., 1998). This makes the precise role of IAA in plant growth promotion difficult to ascertain. Recently, some information has suggested that IAA could also play a role in the case of bacilli PGPR (Idris et al., 2007). This study compared the performance of *B. amyloliquefaciens* FZB42 and four mutants, E*1*01, E102, E103, and E105, with reduced ability to produce IAA. Mutants E101 (Δ*trpBA*) and E102 (Δ*trpED*) were tryptophan auxotrophic, while E103 (Δ*ysnE*, putative IAA transacetylase) and E105 (Δ*yhcX*, putative nitrilase) bore knockout mutations in genes probably involved in IAA metabolism. In all cases, the concentration of the hormone in the culture filtrates was diminished, only reaching 14.7 (E101), 38.0 (E102), 28.9 (E103), and 51.4% (E105) of the IAA produced by the wild-type strain. Addition of diluted culture filtrates of these strains to an *in vitro* experimental system using the aquatic plant *Lemna mimnor* showed a reduced plant growth-promoting effect of those filtrates from the mutants. The percentage of growth promotion reached in plants treated with filtrates from mutants, in comparison with the wild-type FZB42, was 16.8 for E101, 19.2 for

E102, 19.1 for E103,, and 81.5% for E105.These results showed that three of the mutants were highly reduced in their capacity to promote plant growth, which could be related to the reduction in IAA production. Further research is needed to obtain to understand the significance of this mechanism in plant-soil systems and the conditions in which it functions in natural environments.

Other hormones such as gibberellins (Gutiérrez-Mañero et al., 2001; Joo et al., 2005; Joo et al., 2004) and cytokinins (Krebs et al., 1998; Timmusk, 2003; Timmusk et al., 1999; Timmusk and Wagner, 1999) have also been suggested to contribute to plant growth promotion by bacilli. However, all the information supporting this linkage is still quite indirect (see Table 1.4) and more research is required. Two special cases are the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Ghosh et al., 2003) and volatiles (Ryu et al., 2003a), which have not been sufficiently investigated yet but there is strong evidence of their potential role as PGPR-mechanisms in bacilli. ACC deaminase is an enzyme linked to root elongation and amelioration of stress caused by biotic and abiotic factors (Glick et al., 2007). It functions by degrading ACC, a precursor of ethylene in plants, thus producing α-ketobutyrate and ammonia, which in turn lowers the ethylene levels in plant tissue. This reduction of ethylene stimulates plant growth because 1) ethylene is an inhibitor of root elongation and 2) it ameliorates plant stress as ethylene is a key component of the signal transduction pathway (Glick et al., 1998). Although ACC deaminase production seems not to be common in bacilli (Glick et al., 2007), studies developed by Ghosh et al. (2003) reported that three strains of *Bacillus circulans*, *B. firmus*, and *B. globisporus* produced it. This was determined by measuring the amount of α-ketobutyric acid generated from the cleavage of ACC, which indicates the enzymatic activity. Subsequently, these

isolates increased shoot and root growth when inoculated as either seed or soil treatment, whereas an ACC deaminase non-producing *B. subtilis* isolate failed to promote growth. On the other hand, Ryu et al. (2003a) found that the strains *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 produce the volatiles 2,3-bitanediol and acetoin, as well as to promote *Arabidopsis* growth. Plants were stimulated by the bacterium using petri dishes containing a center partition in order to keep the bacterial strain and the plant separate, but sharing a common atmosphere. Likewise, plant growth promotion was observed when exposing the plants to synthetic 2,3-butanediol and to extracted bacterial volatiles from GB03, IN937a, and the model strain 168, which also produce volatile organic compounds. In contrast, exposure to volatiles released by *Escherichia coli* DH5α and mutant strains of *B. subtilis* 168 defective in production of 2,3-butanediol failed to promote growth of *Arabidopsis*.. Similarly, *Bacillus megaterium* UMCV1 has been found to produce acetoin and promote plant growth and root-architectural alterations in *Arabidopsis*, probably involving auxin- and ethylene- independent mechanisms as suggested by tests using the hormone mutant plants *aux1-7*, *axr4-7*, *eir1*, *etr1*, *ein2*, and *rhd6* (Lopez-Bucio et al., 2007). These results strongly suggest a role for these compounds in plant-bacilli interactions, but more studies are required to establish their significance in natural conditions such as those involving soil systems.

5. Other Considerations and Future Perspectives

As argued in this review, if the objective is to take PGPR technology to grower's hands, bacilli constitute a viable alternative; however, in order to overcome the existing inconsistencies in their field performance, a more complete understanding of their mechanisms of action is

required. Knowing as precisely as possible how bacilli-PGPR strains exert their beneficial effects when this takes place would be a good strategy to predict and optimize their effects upon inoculation. Additionally, other advantages could be derived from this knowledge. For instance, it would facilitate finding genetic markers that allow selective isolation of bacilli strains with PGPR properties (Joshi and McSpadden Gardener, 2006; Zhang et al., 2006). In addition, this knowledge would allow improvement of already isolated strains by genetically engineering them to over-express one or more of these traits (Glick and Bashan, 1997). However, this perspective must also include ecological considerations such as root colonization and the physiological stage of the cell after introduction into the rhizosphere. These aspects, even though they are out of the scope of this review, are briefly mentioned next, as the success of PGPR inoculation will surely depend on them as well.

Clearly, the ecology of bacilli inoculants is not well understood yet. However, many valuable investigations have been conducted at this regard. Effective root colonization has been demonstrated for many beneficial bacilli isolates, including well known PGPR strains such as *B. cereus* UW85 (Halverson et al., 1993), *B. subtilis* GB03 (Fukui et al., 1994; Kokalis-Burelle et al., 2006; Mahaffee and Backman, 1993), *B. amyloliquefaciens* IN937a (Kokalis-Burelle et al., 2006), *B. subtilis* MBI600 (Schmidt et al., 2004), *P. polymyxa* B1, B2 (Timmusk et al., 2005), B5 and B6 (W.M. Haggag, 2008), and *B. subtilis* RB-14 (Szczech and Shoda, 2005) among others (Chu et al., 2006; Maplestone and Campbell, 1989; Tokuda et al., 1995; von der Weid et al., 2005; Yan et al., 2002; Young et al., 1995). An important consideration is the use of new tools to study colonization, *e.g.* molecular markers (*luxAB*, *gfp*) and fluorescent *in situ* hybridization. Molecular markers can be very useful, but should be used with care as other

changes in the phenotype can be introduced affecting the actual performance of the strain, which has already been shown in experiments conducted with *B. subtilis* MBI600 (Knox et al., 2002). Nevertheless, colonization studies are an essential part of characterizing PGPR inoculants and understanding their performance in the field (Fall et al., 2004; Reva et al., 2004).

No doubt colonization by plant growth-promoting bacilli is a complex process, where many factors have been found to be involved. For instance, surfactant production has been demonstrated to be essential for plant-associated bacilli to form biofilms (Hsueh et al., 2007). Likewise, aspects such as proteolytic activity (Bindel Connelly et al., 2004) or K^+ concentration (Kinsinger et al., 2005) can play central roles in the resulting colonization, as they are essential for *Bacillus* biofilms. In general, internal factors have been shown to determine the capacity of bacilli strains to colonize, a process that involves several genes (Branda et al., 2004) and seems to be even more complex in plant-associated strains (Chen et al., 2007a). There are also data suggesting that external factors also regulate these processes in bacilli-PGPR. *Bacillus cereus* UW85, for instance, is inhibited by alfalfa exudates so that the plant directly affects the colonizing performance of this strain (Emmert et al., 1998). Similarly, other groups of microorganisms, like nematodes (Knox et al., 2003) and other bacterial taxa (Peterson et al., 2006), have been found to interact with bacilli in the rhizosphere affecting the population dynamics and distribution. Knowledge of these aspects and broader topics such as population ecology (Garbeva et al., 2003; McSpadden Gardener, 2004) are allowing bacilli researchers to reach a better understanding of plant-soil-bacilli interactions.

Finally, it is important to mention an aspect that has been poorly considered in the use of bacilli inoculants: the bacterial physiological stage. The general assumption is that, once the inoculum reaches the spermosphere or the rhizosphere, spores will germinate and metabolically-active vegetative cells will exert their beneficial traits. However, there is little evidence available which supports this model. For example, investigations developed by Vilain et al. (2006) showed that several isolates of *B. cereus* behaved differently in soil than in nutrient-rich media. In soil, bacilli isolates form multicellular structures and sporulate abundantly and easily, while regular laboratory medium did not support formation of the mentioned structures and produced a lower number of spores. These phenomena, accompanied by the observation that the bacterium had the capacity of translocating through soil, suggest the need for studies in more realistic conditions, so that artifacts can be avoided and the natural bacilli behavior can be better illustrated. Likewise, other factors that seem to affect the spore germination rate and cell physiology of bacilli in soil are the inoculum size (Caipo et al., 2002) and nitrate availability (Caldeira et al., 2008; Knox et al., 2000), respectively. This indicates that aspects that are not usually taken into account could be playing important roles. The importance of these considerations is evident for practical applications, as demonstrated by Szczech and Shoda (2006) who found that, while soil application of *B. subtilis* RB14 effectively protected tomato seedlings from *Rhizoctonia*-damping off, seed treatment and combined (soil+seed) treatment did not. These results could not be satisfactorily explained by colonization and antibiotic concentrations, revealing that complex processes should be involved. Questions about the population biology and physiology might be pertinent and useful.

Chapter 2. Plant Growth Promotion by *Bacillus amyloliquefaciens* FZB45 Depends on Inoculum Rate and P-Related Soil Properties

1. Introduction

Mechanisms by which plant growth promoting rhizobacteria (PGPR) exert their benefits have been a research subject for many years (Glick et al., 1999). Understanding those mechanisms would help overcome the variability of PGPR inoculation when plants are grown in soil (Compant et al., 2005; Richardson, 2001) which is a major limitation for using PGPR in agriculture (Siddiqui, 2006; Vessey, 2003). The knowledge of mechanisms and their interactions with soil properties would allow researchers to meet the conditions for optimal results and to predict the outcome, directly impacting practical PGPR use. This approach is particularly important for inoculants based on strains of aerobic endospore-forming bacteria (AEFB), which represent most of the PGPR-based products that are commercially available. There is considerable commercial interest in these bacteria, also referred to as bacilli, because of their high feasibility to be formulated (Mathre et al., 1999; Ongena and Jacques, 2008). This unique formation of highly resistant endospores allows the development of products with a shelf-life of

over one year, which is generally required for biological seed treatments and successful integration in the agricultural market (Driks, 2004; Kloepper et al., 2004).

Several bacterial traits such as production of phytohormones and increased plant nutrient uptake are commonly referred to as putative PGPR-mechanisms in bacilli. For instance, many PGPR strains, including AEFB, have been found to be producers of auxins (Asghar et al., 2002; Lebuhn et al., 1997; Srinivasan et al., 1996), gibberellins (Bottini et al., 2004; Joo et al., 2005; Joo et al., 2004), and cytokinins (García de Salamone et al., 2006; García de Salamone et al., 2001; Timmusk et al., 1999), plant hormones which have been proposed as being linked to their beneficial effect. However, evidence supporting the actual role of those hormones in plant growth promotion under natural conditions is still scarce. In this regard, some studies suggest a biological significance for bacterially-produced indole-acetic acid (IAA) in plant growth promotion by bacilli, *Bacillus amyloliquefaciens* FZB42 (Idris et al., 2007), and non-bacilli strains, *Pseudomonas putida* GR12-2 (Patten and Glick, 2002). In those studies, mutant derivatives with reduced IAA production were less efficient than the wild-type strains to stimulate plant growth under gnotobiotic conditions, which suggests that IAA was a mechanism of growth promotion. Nevertheless, the performance of such mutants in the presence of soil is still to be explored, and the functionality of bacterially-produced IAA in natural environments remains unclear. Further evidence for the biological significance of IAA in plant-PGPR interactions has been provided with *Azospirillum brasilense* Sp245 on wheat (Dobbelaere et al., 1999). In this system, higher bacterial concentrations caused a dramatic reduction in root length and increases in root hair formation. This phenomenon was related to high IAA contents in the

rhizosphere as it was not observed when using a mutant with a 90% reduction in synthesis of this hormone.

Regarding increased plant nutrient uptake, some studies suggest a role for bacilli in nitrogen fixation (Beneduzi et al., 2008a; Beneduzi et al., 2008b; Çakmakçi et al., 2006) and potassium solubilization (Sheng and He, 2006). However, the vast majority of the research in this respect focuses on solubilization of phosphorus (Goldstein and Krishnaraj, 2007; Gyaneshwar et al., 2002; Richardson, 2001; Rodriguez and Fraga, 1999). This is probably due to the importance of phosphorus (P) as a plant nutrient and its frequent presence in soil as unavailable forms (Rodriguez and Fraga, 1999). Considerable amounts of P are present in soils as part of large organic molecules or precipitated as insoluble inorganic complexes with calcium, free oxides, or hydroxides of aluminum and iron; none of which can be taken up by the plant (Rodriguez and Fraga, 1999). Likewise, inorganic P (P_i) is also adsorbed to the surfaces of clays and organic matter, which also reduces the availability of this element for the plant (Fox, 1981). Both precipitation and adsorption of P_i in soil, together designated with the term sorption, are complex reactions and are governed by pH, mineral composition, and texture of the soil (Celi and Barberis, 2007).

A considerable portion, up to 50 to 80%, of total soil P is present as organic molecules (Richardson et al., 2007). One of the most abundant P-containing organic molecules is phytate (also referred to as phytic acid or inositol phosphate), which can account for 20 to 50% of soil organic P (Richardson et al., 2007). Phytate is considered to be a major form of P in soil and, therefore, an important source of this element in the P cycle in soil-plant systems (Richardson et

al., 2007). Based on the annual production of crop seeds and fruits, the main source of phytate, it is estimated that the amount of P existing as phytate could be equivalent to two-thirds of the P applied each year in fertilizers (Mullaney and Ullah, 2007). However, plants neither produce significant amounts of phytate-degrading enzymes (phytases) nor can directly use phytate as a source of P, and thus, they depend on microorganisms to hydrolyze the molecule (Richardson et al., 2007).

Several studies have suggested that phytases could be an alternative for solubilizing phosphorus from phytate in soil and making this element available for plant uptake (Richardson, 2001). Those investigations have included different approaches such as addition of purified microbial phytase (Findenegg and Nelemans, 1993), inoculation with phytase-producing fungi (Tarafdar and Marschner, 1995) or bacteria (Idriss et al., 2002; Richardson et al., 2001b; Unno et al., 2005), and the use of transgenic plants expressing phytases of fungal (George et al., 2005c) and bacterial (Yip et al., 2003) origin. Remarkably, most of the experiments involved in this research have been conducted in the absence of soil, and when done in its presence, plant responses are less consistent and largely dependent on soil type (Richardson, 2007). When this variability is seen following inoculation with phytase-producing microorganisms, common explanations that are frequently given include problems in root colonization or changing environmental conditions. However, a considerable body of information also suggests that P-related soil properties such as P and phytate content, phosphorus-fixation capacity, and pH have a major influence (George et al., 2005a; George et al., 2005b; George et al., 2006; Richardson, 2007). The details and extent of this influence are still poorly understood, especially in the case

of phytase-producing PGPR, where most of these aspects remain to be explored (Richardson, 2007; Richardson, 2001).

Regarding bacilli-PGPR, Idriss et al. (2002) provided evidence that phytase activity of *Bacillus amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation. Experiments were conducted using a soilless gnotobiotic system consisting of corn seedlings grown in a nutrient solution containing phytate as the only source of P and in presence of bacterial culture filtrates. Plant growth was promoted only when culture filtrates of the wild-type strain were added to the system, but not with those of a mutant strain (FZB45/M2) lacking phytase activity. These observations clearly indicated that, in the gnotobiotic system, phytase production was the main mechanism by which FZB45 promoted plant growth. However, those authors cautioned that this study should be considered as a starting point and called for verification under conditions that better mimic the natural environment.

Using PGPR in agriculture will obviously involve the presence of soil, which is expected to affect the bacterial physiology (Vilain et al., 2006), phytase behavior (George et al., 2005b), and the availability of phosphate and phytate (Celi and Barberis, 2007; Celi et al., 1999; Fox, 1981). Yet at the same time, culture filtrates of FZB45 can cause bending of corn coleoptiles (Krebs et al., 1998), suggesting that a direct effect of FZB45 cells could also be expected. If this is the case, aspects such as type and concentration of inoculum could have an important influence on the final effect by this strain, as seen in other systems where direct mechanisms are involved (Dobbelaere et al., 1999). Therefore, when using PGPR in natural conditions, all these factors will determine the actual role of bacterial phytase activity and the final plant growth response.

The aim of the present report was to explore the potential of the phytase-producing strain *B. amyloliquefaciens* FZB45 to promote plant growth and phosphorus uptake in a soil-plant system. For this purpose, the response of Chinese cabbage to inoculation with FZB45 was tested in an experimental system using soil. Plant growth and P content were evaluated under different regimens of soil P_i and phytate, both in relation to soil P-fixing capacity, and with different inoculum concentrations. Evaluation of these factors is a required step for a successful implementation of PGPR as biofertilizers in agriculture (Banerjee et al., 2006; Richardson, 2001).

2. Materials and methods

2.1. Soil characterization

The B horizon of a never-fertilized ultisol from Alabama (U.S.A.) was selected because of its naturally low P content and low level of organic matter. Soil analysis was routinely done in the Soil Testing Laboratory at Auburn University. Analysis of texture classified this soil as loamy with light clays. Chemical analysis, which was performed using Mehlich-1 extract and determination by inductively coupled argon plasma spectrophotometry (ICAP), showed the following parameters: pH 6.1, organic matter 1.4%, and nutrient content (mg kg^{-1}): P = 3, K = 17, Mg = 55, and Ca = 440. Dolomitic lime (1 g kg^{-1}) was added to the soil in order to increase Ca and Mg contents and reaching pH 6.5. A mixture 1:1 (w/w) soil:sand was used for all the experiments. A P-sorption curve establishing the relationship between P applied and P in soil

solution was prepared for this mixture (Fox and Kamprath, 1970b; Nair et al., 1984). For this purpose, both 1.5 g of 4 mm-sieved air-dried soil and 1.5 g of sand (3 g soil mixture total) were put into 50 ml polypropylene tubes. Thirty ml of 0.01 M $CaCl_2•2H_2O$ containing proper amounts of KH_2PO_4 were added into separate tubes in order to reach concentrations equivalent to 0, 5, 10, 15, 20, 25, 30, 40, and 50 mg P kg^{-1} soil. Two drops of chloroform were added into each of the tubes which were then placed horizontally in an orbital shaker for 24 h at room temperature and 150 rev min^{-1}. After incubation, samples were centrifuged for 5 min at 3000 rev min^{-1} and filtered through filter paper Whatman No. 1. Phosphorus in solution was determined using molybdate-blue method (Murphy and Riley, 1962).

2.2. Bacterial inoculum preparation

The strain *Bacillus amyloliquefaciens* FZB45 (Idriss et al., 2002) was kindly provided by Dr. Rainer Borriss (Institut für Biologie, Humboldt Universität, Berlin). A bacterial spore suspension was prepared by growing the strain on a modified medium to stimulate sporulation. One liter of medium contained proteose peptone (vegetable, Fluka), 3.3 g; beef extract powder, 1.0 g; D-lactose monohydrate, 5.0 g; NaCl, 5.0 g; K_2HPO_4, 2.0 g; KCl, 1.0 g; $MgSO_4•7H_2O$, 0.25 g; $MnSO_4$, 10 mg; and agar, 18 g. After incubation for 7 days at 28ºC, spores were harvested from medium surface using 5 ml of sterile distilled water (SDW) per Petri dish. This suspension was centrifuged at 3000 rev min^{-1} for 10 min, the supernatant was discarded, and the pellet was resuspended in SDW. This suspension was finally pasteurized for 15 min at 80ºC and its concentration was determined by plate counting. The spore suspension was stored at 4ºC until use, and the spore viability was confirmed at the moment of inoculation.

2.3. Soil-plant experiment

Two-day old seedlings of Chinese cabbage (*Brassica rapa* Kaboko Hybrid, Park Seed Co., Greenwood, S.C. 29647) were used in all the experiments. Seeds were disinfected by soaking in 70% ethanol for 1 min, rinsing once with SDW, then soaking again in 0.5% sodium hypochlorite for 10 min, and finally rinsing 15 times with SDW. Disinfected seeds were transferred to Petri dishes with 2% water-agar and incubated for 2 days at 28ºC in the dark, until germination occurred. Then, 2-day old seedlings were planted individually in plant growth containers which consisted of 50 ml-plastic centrifuge tubes containing 70 g of a mixture 1:1 (w/w) soil-sand. The amount of soil (air-dried and sieved at < 4mm) and sand (35 g each) to be added into each container were weighed separately and then mixed in order to ensure homogeneity. Likewise, containers were fertilized individually with 5 ml of distilled water containing NH_4NO_3, $Ca(NO_3)_2 \bullet 4H_2O$, $MgSO_4 \bullet 7H_2O$, and KCl in equivalent amounts to add 100 mg N, 100 mg Ca, 60 mg Mg, and 85 mg K kg^{-1} soil. The experiment was conducted following a 4 x 3 factorial arrangement. Four different P regimens were evaluated: no addition of P, 15 mg P kg^{-1} soil, 74.57 mg phytate kg^{-1} soil (equivalent to 15 mg P kg^{-1} soil, 1X), and 447.43 mg phytate kg^{-1} soil (equivalent to 90 mg P kg^{-1} soil, 6X). P amendments were applied to each container using 5 ml of distilled water containing the respective amount of NaH_2PO_4 (source of P_i) or phytic acid dodecasodium salt hydrate from rice (Sigma P0109). Bacterial treatment was achieved at three levels: no inoculation (only SDW) and two rates of FZB45, 10^6 and 10^8 spores $seedling^{-1}$ carried in 0.1 ml suspension. Five containers, each having a 2-day old seedling, were used for each combination treatment, and the experiment was conducted twice. Soil moisture was kept between 40-60% of maximum water holding capacity, and plants were incubated at 25ºC in a growth

chamber with 16 h light and 8 h darkness for 14 days. Response variables were fresh shoot weight (FSW), shoot inorganic P content (shoot P_i content), inorganic P concentration (P_i concentration, based on fresh weight), and total P concentration (P_t concentration, based on dry weight).

2.4. Plant P extraction and determination

Whole plant P_i, which is considered to be a highly sensitive index for P nutrition, was extracted following the method by Huang et al. (2005b). For this purpose, the shoot of each plant was washed in distilled water and put into 50 ml-plastic centrifuge tubes containing 15 ml 0.1 M H_2SO_4. Samples were agitated in an orbital shaker for 16 h at 160 rev min^{-1} and 25ºC, then heated in a water bath at 85ºC for 15 min, and cooled down for P_i determination. P in solution was determined in the extractant by the molybdate-blue method (Murphy and Riley, 1962) using a P standard curve also prepared in 0.1 M H_2SO_4. P_i was expressed as shoot P_i content (µg P_i $plant^{-1}$) and P_i concentration (mg P_i kg^{-1}, based on fresh weight). For P_t content, determination was done in leaf disks according to the method described by Aziz and Habte (1987). Briefly, a disk (0.6 cm) was taken from the upper half of the youngest fully opened leaf of each plant and transferred into a 1.5 ml-microcentrifuge tube. Leaf disks were dried overnight at 70ºC, weighed, and then ashed at 500ºC for 3 h. These ashes were dissolved in 10 ml of distilled water and P in solution was determined by the molybdate-blue method (Murphy and Riley, 1962). P_t content was expressed as P_t concentration (%) taking the disk dry weight as the base.

2.5. Gnotobiotic root elongation assay

The effect of two different *B. amyloliquefaciens* FZB45 inoculum sizes on root elongation of Chinese cabbage was tested under gnotobiotic conditions, following the method by Penrose and Glick (2003). Different from the mentioned method, seed disinfection and bacterial inoculation were performed as described above for the soil-plant experiment except that spores were applied on seeds instead of 2-day seedlings. Ten seed germination pouches (cyg, mega international, St. Paul, MN, USA) per treatment, with 5 seeds per pouch, were used. Pouches were maintained in covered transparent plastic boxes at 25ºC in a growth chamber with 16 h light and 8 h darkness. Root lengths were measured 7 days after inoculation discarding those seeds that did not germinate by the second day. This experiment was conducted twice.

2.6. IAA determination

The capacity of *B. amyloliquefaciens* FZB45 to produce IAA was determined *in vitro*. The strain was grown for 24 h in TSB, and 20-µl aliquots were transferred into 50-ml flasks containing 10 ml of TSB supplemented to reach 0, 50, 100, 200, and 500 µg ml^{-1} of L-tryptophan (FisherBiotech, BP395). Tryptophan was added as a filtered-sterilized (0.22 µm) 2-mg ml^{-1} stock solution prepared in warm water (Patten and Glick, 2002). Flasks were incubated at room temperature and 150 rev min^{-1} on an orbital shaker and samples were taken 24, 48, and 72 h after inoculation. $O.D._{630}$ was recorded as an indicator of growth and an aliquot of each flask was centrifuged (8000 rev min^{-1}) to remove bacterial cells. One ml of supernatant was mixed with 4 ml of Salkowski's reagent (150 ml of 18 M H_2SO_4, 250 ml distilled water, 7.5 ml of 0.5 M

$FeCl_3•6H_2O$) and absorbance at 535 nm was measured after 20 min (Gordon and Weber, 1951; Patten and Glick, 2002). IAA concentration was estimated by comparison with a standard curve prepared with Indole-3-acetic acid (Sigma I-2886).

2.7. Statistical analysis

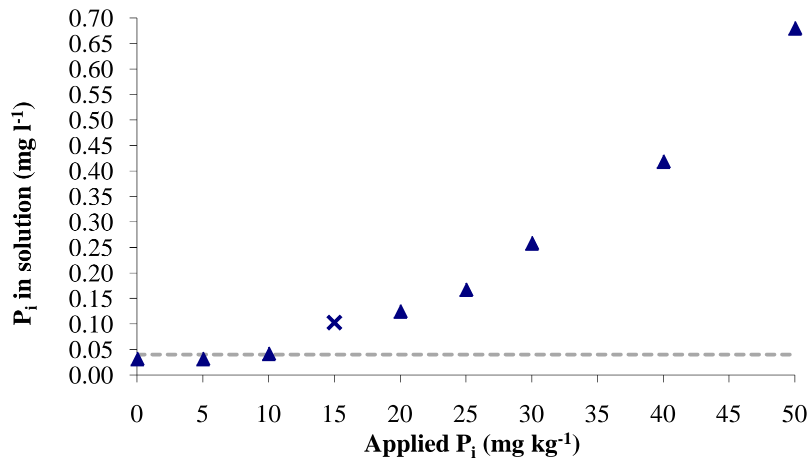
Response data from the two plant-soil experiments were analyzed jointly as a factorial with bacterial treatment and P regimen as fixed effects. The effect for experiment was extracted from the residual error term for FSW and P_t concentration and was treated as a random effect. Normality and equal variances assumptions were first evaluated using the student panel graphs generated by SAS® GLIMMIX Procedure, which was also used for all the analyses. Normal distribution was only warranted for FSW, while all the response variables involving P determination followed a log-normal distribution. As the equal variance assumption was not fulfilled for FSW and P_t concentration, the variance structures for those two response variables were modeled (*R*-side of the covariance parameters of SAS) using the group option to create homogeneous variance groups. A smaller AIC value from the 'information criteria' output and a better graphical residual distribution were considered as indicators of a good fit for the model. The residual term was the pooled residual within bacterial inoculation × P regimen combination variation, as the experimental design was a CRD. For those response variables with a statistically significant interaction, all pairwise simple effect comparisons among bacterial inoculation levels within each P regimen were done using the simulate adjustment of GLIMMIX procedure. Significance classes are presented in the graphs. Root length data from the two gnotobiotic assays were analyzed jointly with bacterial inoculum sizes as the only fixed effect; the effect for

pouch nested within treatment (bacterial inoculum size) was treated separately as a random effect. For these data, normality and equal variance assumptions were fulfilled. Dunnett's test was used to assess the difference between each of the two bacterial rates and the untreated control.

3. Results

3.1. Effect of *B. amyloliquefaciens* FZB45 inoculation in soil

Figure 2.1 Concentration of P in soil solution after application of different amounts of inorganic P to the mixture 1:1 (w/w) soil:sand used in all the experiments



Each point represents one single determination. Dashed line indicates the level of P in soil solution associated with 80-95% of maximum yield of cabbage (*Brassica oleracea*; Hue et al., 2000). In a separate experiment (data not shown), application of 15 mg P_i kg^{-1} soil (diagonal cross point marker) caused a 57% increase in fresh shoot weight of Chinese cabbage (*Brassica rapa* Kaboko Hybrid) two weeks after planting. This level of P fertilization was used as the full fertilized treatment.

The P-sorption curve (Figure 2.1) indicated that 15 mg kg^{-1} was the minimum amount of P_i needed to be applied in order to reach 0.04 mg l^{-1} of P_i in soil solution, which is considered the

minimum concentration required to support suitable growth of cabbage (*Brassica oleracea*; Hue et al., 2000). Based on this information, the response of Chinese cabbage to P fertilization in the experimental soil used was tested (data not shown). This experiment showed that application of 15, 30, and 60 mg P_i kg^{-1} soil caused plant growth increases of 57, 77, and 112%, respectively, compared with a non-fertilized control. Taking the previous information into account, application of 15 mg P_i kg^{-1} soil was used as the full fertilized treatment for the following experiments.

Table 2.1 *P*-values from the analysis of variance for the effect of *B. amyloliquefaciens* FZB45 inoculation, different P regimens, and their interaction on fresh shoot weight, inorganic (P_i), and total (P_t) phosphorus content of Chinese cabbage grown in soil for 2 weeks under growth chamber conditions

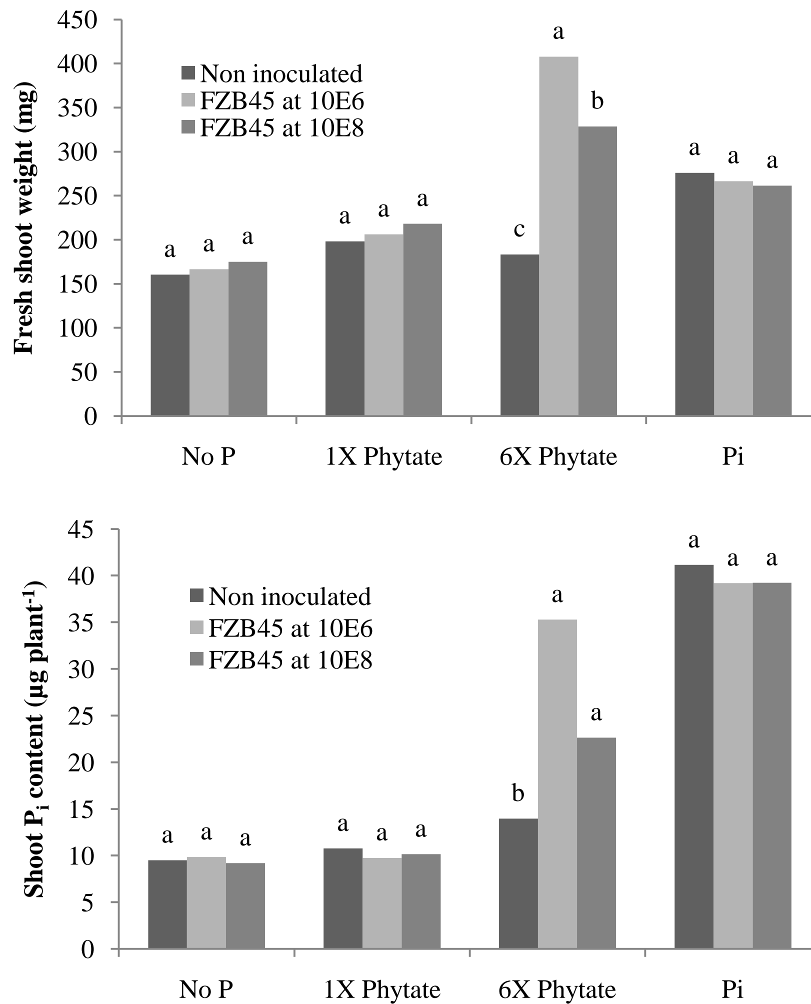
Factor	Fresh shoot weight	Shoot P_i content	P_i concentration	P_t concentration
P regimen	0.011*	< 0.001*	< 0.001*	< 0.001*
Bacterial inoculation	< 0.001*	0.109	0.716	0.752
P regimen*Bacterial inoculation	< 0.001*	0.012*	0.782	0.222

Bacterial inoculation was done at two different rates (10^6 and 10^8 spores $seedling^{-1}$). P regimens included application of P_i and two rates of phytic acid. Data are from two separate experiments, each having five replicates per treatment combination. *P*-values followed by an asterisk are considered to be statistically significant.

For the soil-plant experiment, factorial analysis revealed a highly significant interaction between P regimen and bacterial inoculation on both FSW ($P < 0.001$) and shoot P_i content ($P = 0.012$; Table 2.1). Specifically, bacterial inoculation caused a significant increase of FSW and shoot P_i content only at the higher rate of phytate (6X, which corresponded to 447.43 mg phytate kg^{-1} soil, equivalent to 90 mg P kg^{-1} soil); whereas no effect was observed under the other three P regimens tested (Figure 2.2). Although both rates of inoculums caused significant increases with

respect to the non-inoculated control, the lower rate (10^6 spores seedling^{-1}) was superior than the higher (10^8 spores seedling^{-1}) for shoot fresh weight (P = 0.020) and plant P_i content (P = 0.092).

Figure 2.2 Effect of the inoculation with two different rates of *B. amyloliquefaciens* FZB45 under four different P regimens on fresh shoot weight and plant inorganic P content of Chinese cabbage grown in soil for 2 weeks under growth chamber conditions



Averages expressed as the least squares means. Those with the same letter within the same P regimen are not significantly different (P-value > 0.05) according to simulate adjustment of GLIMMIX procedure. Interactions were significant for both response variables and P-values for the F-tests are shown in Table 2.1. Inoculations were performed as spore suspensions and are expressed as spores seedling^{-1}. Data are from two separate experiments, each having five replicates per treatment combination. Plant P_i content was analyzed as $\log_e$-transformed data and had 101 degrees of freedom, while fresh shoot weight had 97.

Neither bacterial inoculation nor its interaction with P regimen had a significant effect on P_i and P_t concentrations, even though plant growth and shoot P_i content were significantly increased (Table 2.1). In contrast, P regimen had a significant effect on all the response variables evaluated (Table 2.1). However, when compared in the absence of bacteria, only the addition 15 mg P_i kg^{-1} soil caused significant increases in FSW ($P < 0.05$), shoot P_i content ($P < 0.001$), and Pi concentration ($P < 0.01$). The other two P regimens, which corresponded to the addition of two different rates of phytate, did not significantly affect any response variable ($P > 0.05$).

3.2. Gnotobiotic root elongation assay and IAA production

In order to elucidate any direct effect of FZB45 on root development that could explain the higher increases observed with the lower inoculum concentration, a gnotobiotic assay was conducted in sterile seed germination pouches. In effect, only the lower bacterial rate (10^6 spores $seed^{-1}$) significantly promoted root elongation of Chinese cabbage one week after inoculation ($P = 0.007$), whereas the higher rate (10^8 spores $seed^{-1}$) showed no difference compared to the non-inoculated control ($P = 0.486$; Table 2.2). Such soil independent root growth-promoting effect at low bacterial concentrations suggests the involvement of a hormone-like compound, probably auxin type. To further clarify this, we determined if *B. amyloliquefaciens* FZB45 was able to produce IAA, a widely known bacterially-produced auxin. This strain was effectively able to produce this compound *in vitro*, which was additionally stimulated by the presence of tryptophan in the growth medium (Table 2.3). Even in absence of any tryptophan amendment, IAA was detected in the medium after 48 h; however, production of this compound was increased with addition of more than 200 µg of tryptophan ml^{-1}, being detected as soon as 24 hours after

inoculation of the medium. In general, at all the three reading times, the addition of tryptophan to the medium stimulated a higher production of IAA, reaching five to six fold increases for the highest level tested, 500 µg of tryptophan ml^{-1}.

Table 2.2 Least squares means (LS mean), standard errors (SE), and probability of difference to the untreated control (Dunnett's test) for two concentrations of *B. amyloliquefaciens* FZB45 on root length of Chinese cabbage under gnotobiotic conditions one week after seed inoculation

Treatment	LS mean	SE	Dunnett's *P*-value
Control	6.85	0.26	
FZB45 at 10E6	7.97	0.26	0.007
FZB45 at 10E8	7.23	0.25	0.486

Inoculations were performed as spore suspensions and are expressed as spores $seed^{-1}$. Data are from two separate experiments, each having five plants per pouch and ten pouches per treatment. *F*-test had a $P = 0.011$ and degrees of freedom for error = 56.

Table 2.3 Production *in vitro* of IAA by *B. amyloliquefaciens* FZB45 in the presence of various concentrations of tryptophan

Tryptophan concentration	IAA production (µg ml^{-1} OD_{630}^{-1} unit)		
(µg ml^{-1})	24 h	48 h	72 h
0	0.0 ± 0.0	0.6 ± 0.0	0.8 ± 0.0
50	0.0 ± 0.0	0.9 ± 0.3	1.1 ± 0.2
100	0.0 ± 0.0	1.2 ± 0.1	1.7 ± 0.1
200	0.4 ± 0.2	1.6 ± 0.1	2.3 ± 0.1
500	0.5 ± 0.1	3.4 ± 0.4	4.2 ± 0.3

Average ± standard error from three replications.

4. Discussion

In our system, *B. amyloliquefaciens* FZB45 promoted plant growth and P nutrition in soil. These effects, however, only occurred under conditions conducive for phytase activity, supporting the conclusion that phytase activity is the major mechanism for plant growth promotion for this strain. Additionally, FZB45 exerts a direct mechanism on plant growth, probably IAA production, which creates a concentration-dependent response to inoculation and interacts with phytase-mediated effect.

Unexpectedly, the experiments conducted by Idriss et al. (2002) using corn seedlings (*Zea mays* cv. Elita) grown in nutrient solution and inoculated with FZB45 spores could not be reproduced in our laboratory (data not shown). Following the protocol of Idriss et al., FZB45 spores were applied to corn seedlings at three different concentrations of phytate (0.3, 1.0, and 4.0 mM), but in our tests no response to inoculation was observed and the addition of phytate to the nutrient solution resulted in chlorosis and severe symptoms of calcium deficiency on the tested corn hybrid. In addition, a white precipitate of phytate and $Ca(NO_3)_2 \bullet 4H_2O$ (a component of the nutrient solution) was evident at 1 and 4 mM phytate concentrations. Inositol phosphates possess a high chelating power, especially for divalent cations such as Ca^{+2}, Mg^{+2}, and Zn^{+2}, which would explain the precipitate formation (Grynspan and Cheryan, 1983). The different results obtained in the tests by Idriss et al. and in our laboratory may partially be explained by the use of different corn hybrids. Possibly the hybrid used in our tests (DKC61 73) was more sensitive to calcium deficiencies or to the components in the liquid medium. These observations, along with a low response to P fertilization in the soil used in our experiments (8, 18, and 22%

for 15, 30, and 60 mg P kg^{-1} soil, respectively), caused us to reject the use of corn as the model plant for our experimental system. Chinese cabbage proved to be an excellent choice because of its fast and homogeneous growth as well as its high suitability for P nutrition assays due to the small size of seeds and sensitive response to changes in soil P availability (Huang et al., 2005b). Additionally, unlike corn and tomato (which was also highly responsive to P addition), Chinese cabbage is independent of mycorrhizal associations (Habte, 2000). This feature eliminates any potential interference caused by mycorrhizal fungi which aid in plant uptake of soil P.

Our results support the conclusion that phytase activity is the major mechanism by which *B. amyloliquefaciens* FZB45 promotes plant growth. No effect of inoculation was observed without phytate addition, neither under P-limited conditions nor full P fertilization (Figure 2.2). Thus, under our experimental conditions, FZB45 only increased the growth of Chinese cabbage if phytate was available in soil. This behavior, along with the fact that P was the only limiting nutrient in soil, strongly suggests that the beneficial effect of inoculation was due to P solubilization through the strain's phytase activity. This also indicates that any other beneficial trait of FZB45 was secondary and not biologically significant in the absence of phytate, regardless if P_i content in soil was high or low. It is important to mention that phytase activity could also increase the availability of phytate-chelated nutrients such as Ca^{+2}, Mg^{+2}, or Zn^{+2}, an effect that cannot be ruled out. However, this is an unlikely explanation for the observed growth response as soil fertilization was previously standardized to have sufficient elements (except P), and no deficiency symptoms were observed for any of those elements, even when phytate was added.

Phytase activity has been shown to solubilize sufficient P to increase plant growth (Richardson, 2001). Reports include different approaches such as addition of purified microbial phytases (Findenegg and Nelemans, 1993), inoculation with phytase-producing microorganisms (Idriss et al., 2002; Richardson et al., 2001b; Tarafdar and Marschner, 1995; Unno et al., 2005), and the use of transgenic plants expressing microbial phytases (George et al., 2005c; Yip et al., 2003). Among these reports, plant growth increases by inoculation with phytase-producing bacteria are the fewest so far. To our knowledge, besides the previously mentioned study with FZB45 on corn in nutrient solution (Idriss et al., 2002), only three other reports have linked plant growth promotion with bacterial production of phytase. Two of these reports, both in soilless systems, used bacterial strains with natural phytase production: *Pseudomonas* sp. CCAR59 on *Trifolium subterraneum* (Richardson and Hadobas, 1997; Richardson et al., 2001b) and several strains of *Burkholderia* spp. on *Lotus japonicus* (Unno et al., 2005). The third report used the genetically-modified strain *Bacillus mucilaginosus* NKTS-3, which expresses a phytase of fungal origin (Li et al., 2007). In this study, which was conducted in soil, both NKTS-3 and its wild-type strain (non-phytase producer) promoted tobacco growth and P content. However, the effect of the phytase-producing strain was significantly higher.

Studies of phosphorous solubilization in general, including phytase activity, should take into account fundamental principles of soil fertility. For example if plant growth is to be promoted through increases in P uptake, native soil P content should be low enough for the plant to respond to its increase. Additionally, the content of this element must be the only limiting factor for plant growth. Some of the failures in promotion of plant P uptake by P-solubilizing PGPR are found to be associated with soil P contents sufficiently high to support satisfactory plant growth

(de Freitas et al., 1997) or with the presence of an additional limiting factor which hinders the response to soil P increases (Fernández et al., 2007). In our study, soil P content and plant response to addition of P_i were previously characterized in order to accomplish these two soil fertility principles. Likewise, all the other nutrients were supplied to ensure their sufficiency and rule out the presence of any additional limiting factor for plant growth in the system. Additionally, phytate itself was not a significant source of P as indicated by the absence of response to phytate addition when FZB45 was not inoculated. This reveals the incapacity of the plant to use or hydrolyze phytate and a nonsignificant phytase activity by indigenous soil microorganisms.

Another essential consideration in studies of phytase activity as a mechanism for plant growth promotion by PGPR is that soil properties, such as organic matter content, pH, texture, P-fixation capacity, and calcium content will mediate the results. Phytate (substrate; Celi and Barberis, 2007), phytase (enzyme; George et al., 2007a), and phosphate (product; Fox, 1981) interact with soil, and because of this, they behave differently in soil than *in vitro*. First of all, if phytate is to be solubilized by the PGPR, it must be present and available in the soil. Little is known about the amount of phytate that is needed in soil to achieve a plant response to P-solubilization. However, one study by George et al. (2005a), using a transgenic line of *Trifolium subterraneum* expressing a fungal phytase, reported significant increases of plant P content only when plants were grown in a soil with at least 8.7% organic matter content. The native amount of phytate in our experimental soil, although not determined, was expected to be low. This is suggested by the low levels of extractable soil P and organic matter. When phytate was added at 447 mg kg^{-1} soil,

FZB45 promoted plant growth significantly. This amount of phytate is in the range reported for phytate contents naturally occurring in soils (0.3 – 987 mg kg^{-1} soil; Turner et al., 2002).

Concerning the effect of soil properties on phytate availability, it should be noted that phytate, like phosphate, is adsorbed to soil colloids (Celi et al., 1999). The P-fixing capacity of a substrate in relation to phytate availability was previously shown to limit the response of *Trifolium subterraneum* to inoculation with *Pseudomonas* sp. CCAR59 (Richardson et al., 2001b). In this study, when the experiment was conducted with agar slants as a substrate, which has a low P fixation level, plant growth and P content were always significantly increased by inoculation. In contrast, when plants were grown in sand-vermiculite medium, a substrate with high P-fixing capacity, bacterial inoculation was only effective at the highest tested phytate treatment, 155 mg phytate $tube^{-1}$ (equivalent to 2768 mg kg^{-1}). A similar pattern would be expected when natural soil is used. In general, phytate is more easily utilized as P source in those soils having a lower P-fixing capacity (Richardson et al., 2007) or when practices to increase soil phytase-labile P are used (Hayes et al., 2000b). For example, experiments with transgenic plants expressing a fungal phytase gene showed improved P nutrition only in a vertisol but not in any of three alfisols or a spodosol (George et al., 2005a). In our study, fixation to soil clays could explain the absence of response to inoculation at the lower rate of phytate, even though this rate had sufficient P to support satisfactory plant growth.

Regarding the effect of soil properties on phytase, it is known that phytate-degrading enzymes of fungal origin (histidine acid phytases) can also be adsorbed to soil colloids (George et al., 2005b). This affects the enzyme-substrate interaction and the resulting efficiency of phytase. The

behavior of phytases was shown to depend on their physicochemical properties (George et al., 2007b), and thus, specific information for *Bacillus* phytases is required. These phytases possess several particularities that will probably confer on them a different behavior: first, they do not share sequence homology with other phytases (Fu et al., 2008); second, they are classified into a different group (β-propeller phytases; Mullaney and Ullah, 2003); and finally, they require Ca^{2+} for stabilization and activity (Oh et al., 2001). Phytases from *Bacillus* were shown to solubilize sufficient P in soilless systems to increase plant growth significantly. Those studies involved the inoculation of phytase-producing bacilli (Idriss et al., 2002) and the use of transgenic plants expressing *Bacillus* phytases (Lung et al., 2005; Yip et al., 2003). However, soil tests are still required in order to determine the functionality and behavior of this group of phytases in the presence of soil. Our results indicate that the phytase produced by FZB45, a typical β-propeller phytase (Idriss et al., 2002), was active in the soil used, but further characterization is needed to know the actual effect that soil has on this enzyme.

Besides the effects of soil on substrate, enzyme, and product mentioned above, soil will also determine phytase activity by PGPR via changes in bacterial physiology and gene expression. Soil influences the global physiology of the bacterium, making its phenotype different from that observed in laboratory media (Vilain et al., 2006). Likewise, *in vitro* studies have shown that the expression of the *phyC* gene, which encodes for phytase in FZB45, begins during transition from exponential to stationary phase (Makarewicz et al., 2008) and occurs at phosphate concentrations lower than 0.3 mM (9.3 mg P_i l^{-1}; Makarewicz et al., 2006). This means that, for FZB45 to express the *phyC* gene in soil, the strain must be at the right physiological stage and the P_i content should be suitable. The results presented here suggest that these two conditions were met

in our soil, which had 0.04 mg P l^{-1}, and the phytase gene could be expressed. P_i concentrations in natural soil solutions are seldom higher than 0.31 mg l^{-1} (Bieleski, 1973), which would allow the expression of the *phyC* gene; however, more studies should be done in this regard to characterize the expression of this gene and the bacterial physiology in natural environments.

In addition to producing phytase, FZB45 also displayed a direct, soil-independent mechanism for growth promotion of Chinese cabbage. This was suggested by the concentration-dependent effect of inoculation in the plant-soil experiment (Figure 2.2) and confirmed by the root pouch assay (Table 2.2). The best effect at the lower concentration suggests that a hormone-like metabolite could be involved in this effect. Although different phytohormones might be considered, IAA is a highly likely candidate due to its widely known root elongation effect and its role for the concentration-dependent effect of other PGPR (Dobbelaere et al., 1999). Additionally, in this study FZB45 produced detectable amounts of IAA *in vitro* (Table 2.3), and previously, its culture filtrates caused an IAA-like effect on corn coleoptiles (Krebs et al., 1998). However, to test the biological significance of this IAA production, creation of defective mutants will be required. The role of IAA on plant growth promotion by PGPR has been suggested for a long time and it is proven for the strains *Pseudomonas putida* GR12-2 (Patten and Glick, 2002), *Azospirillum brasilense* Sp245 (Dobbelaere et al., 1999), and *Bacillus amyloliquefaciens* FZB42 (Idris et al., 2007).

Facts such as no response to inoculation when phytate was not added and the better response to the lower inoculum rate when phytate was present suggest an interaction between the direct and the phytase-mediated effect. A hypothesis explaining this could be that FZB45's direct effect

promotes root elongation, which allows a greater exploration of soil and increased nutrient uptake. However, particularly in the case of FZB45, this phenomenon is only biologically significant if soil P was limiting and its concentration could be increased via phytase activity. Of course, such a hypothesis must be tested and the interaction between both mechanisms further investigated as very little is known about interactions among mechanisms of plant growth promotion in PGPR.

An ongoing challenge for the widespread use of PGPR-based biofertilizers in agriculture is to increase their consistency of performance. The results of this study demonstrate that by knowing a specific mechanism for plant growth promotion by PGPR, it is possible to predict conditions where plant growth promotion will result. Hence, such knowledge will be an important step to increasing the consistency of growth promotion by PGPR.

Chapter 3. Differentiation of *Bacillus subtilis/amyloliquefaciens* PGPR Strains Based on a Highly Conserved Phytase-Coding Gene

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) are considered to have a great potential as biofertilizers (Banerjee et al., 2006; Vessey, 2003). However, mechanisms by which PGPR exert their benefits are not yet fully understood (Niranjan Raj et al., 2006)., and knowledge of these could facilitate the search for new PGPR strains as well as the prediction and optimization of PGPR performance (Compant et al., 2005; Richardson, 2001). Knowledge of mechanisms is particularly important in the case of PGPR strains of aerobic endospore-forming bacteria (bacilli PGPR), because they represent the majority of PGPR-based products commercially available (Driks, 2004; Kloepper et al., 2004).

One of the mechanisms that has attracted much attention is solubilization of phosphorus (P) (Goldstein and Krishnaraj, 2007; Gyaneshwar et al., 2002; Richardson, 2001; Rodriguez and Fraga, 1999). This is probably due to the importance of P as a plant nutrient and its frequent presence in soil in unavailable forms. These forms include large organic molecules and insoluble inorganic complexes (Raghothama and Karthikeyan, 2005). Reports of P solubilization by rhizosphere bacteria are abundant and have been mainly focused on solubilization of P from

inorganic sources (Rodriguez and Fraga, 1999). Although the contribution of this solubilization to plant P nutrition has not yet been clearly established, its potential is considered to be high, especially when amendments with low soluble P, *e.g.* rock phosphate, are used (Toro et al., 1997).

Regarding solubilization of P from organic molecules, more attention has been given to the role of PGPR in recent years. Depending on the type and management of soil, organic forms of P constitute the predominant fraction of P in the soil solution accounting for around 50% of the total soil P (Richardson et al., 2009). At the same time, among the P-containing organic molecules, phytate (inositol phosphate) is a dominant molecule, accounting for 20 to 50% of soil organic P (Richardson et al., 2007). However, plants do not produce significant amounts of phytases (phytate-degrading enzymes) nor can they use this molecule directly as a source of P. Therefore, phytase-producing microorganisms play an essential role in solubilization of P from phytate, thereby making it available for plant and microbial uptake (Richardson et al., 2007).

The use of microbial phytases has been suggested as an alternative for solubilizing P from phytate in soil and improving plant P nutrition (Richardson, 2001). This has been shown by different approaches such as addition of purified microbial phytase (Findenegg and Nelemans, 1993), inoculation with phytase-producing microorganisms (Tarafdar and Marschner, 1995; Unno et al., 2005), and the use of transgenic plants expressing microbial phytases (George et al., 2005c; Yip et al., 2003). However, the efficacy of phytases from different sources is expected to vary because of their diverse structures and mechanisms of action (Mullaney and Ullah, 2003), which are represented in their classification into four different groups: histidine acid

phosphatases (HAP), β-propeller phytases (BPP), cystein phosphatases (CP), and purple acid phosphatases (PAP) (Mullaney and Ullah, 2007). For instance, phytases from the fungi *Peniophora lycii* and *Aspergillus niger*, which belong to the same HAP group, shared 38% of their amino acid sequence and displayed different levels of activity and stability when added to soil (George et al., 2007b). Such a different behavior of those two phytases was explained by a dissimilar interaction with soil particles and a different response to soil pH (George et al., 2007b).

Bacillus phytases, which are classified as β-propeller phytases (BPP), have shown to solubilize sufficient P to increase plant growth significantly. This was observed in soilless systems with the inoculation of phytase-producing bacilli (Idriss et al., 2002) and the use of transgenic plants expressing *Bacillus* phytases (Lung et al., 2005; Yip et al., 2003). In addition, in the present dissertation (see chapter II), inoculation of the phytase-producing strain *Bacillus amyloliquefaciens* FZB45 promoted the growth and P uptake of Chinese cabbage in soil. This only occurred under conducive conditions for phytase activity, suggesting that FZB45 phytase was active in soil and significantly contributed to the plant growth-promoting effect that was observed. Nonetheless, little is known about the behavior of *Bacillus* phytases in soil and peculiarities such as a their high thermal stability, optimum activity around neutral pH, and dependency on Ca^{+2} (Fu et al., 2008) suggest a different behavior from that observed in other phytases (George et al., 2007b).

Most phytase producing-bacilli strains reported so far have been classified as *Bacillus subtilis* or *Bacillus amyloliquefaciens* (Fu et al., 2008). However, due to the closeness of these two

species and their difficult discrimination (Priest et al., 1987), some reports are confusing with respect to the classification of the strains. For instance, one of the *Bacillus* phytases more widely studied as a model is produced by the strain VTT E-68013, which is assigned in several papers to *B. subtilis* (Kerovuo et al., 2000a; Kerovuo et al., 1998). However, according to the VTT Culture Collection of Technical Research Center of Finland, this strain is currently classified as *B. amyloliquefaciens* (https://culturecollection.vtt.fi:8443/ccdb/index.html, as consulted on June 22, 2010). Other strains, for example, are only identified at the genus level and their species classification is uncertain (Kim et al., 1998a).

Even though *B. subtilis* and *B. amyloliquefaciens* are closely related, important differences occur between these two species. The most important difference is that they share less than 25% of DNA homology, which, opposed to their phenotypic similarity, drove their separation into two different species (Priest et al., 1987). Consistently, genome analysis focused on biocontrol potential has revealed key differences in the type of antibiotics encoded by the genome of each species (Chen et al., 2009a). In ecological terms, *B. amyloliquefaciens* strains were generally better adapted than isolates of *B. subtilis* to colonization of the rhizosphere of three plant species (Reva et al., 2004). This suggests ecotype differences that are still poorly explored and that could be related to substrate use and speciation in these two bacterial groups (Cohan, 2006; Connor et al., 2010).

Little is known concerning phytase-production for most bacilli-PGPR strains. To further explore this subject, the present study aimed to screen for the presence of a phytase-coding gene in selected PGPR strains of the *B. subtilis/amyloliquefaciens* group and to determine whether its

presence was correlated to their taxonomic classification. The presence of the phytase gene was determined by polymerase chain reaction (PCR) using specific primers for the *phyC* gene. Subsequent characterization of the gene was conducted by sequencing and comparative analysis of the sequences. Finally, the evolutionary relationship among the strains was established through a phylogenetic analysis of the 16s rRNA, *gyrA*, and *cheA* gene sequences.

2. Materials and Methods

2.1. Bacterial strains and DNA extraction

All bacilli strains used in the present study previously showed plant growth-promoting activities, and some of them are included in commercial products (Table 3.1). Strains *B. amyloliquefaciens* FZB24, FZB42, and FZB45 were kindly provided by Dr. Rainer Borriss (Institut für Biologie, Humboldt Universität, Berlin). Strains *B. subtilis* UA321 and UA322, isolated from chrysanthemum rhizosphere, were provided by the Microbiological Control Laboratory at the University of Antioquia (Medellín, Colombia). The rest of the strains belonged to the plant-associated bacilli collection of the Phytobacteriology Laboratory at Auburn University and had been previously identified as members of the *B. subtilis/amyloliquefaciens* group based on their fatty acid profile (FAMES) or 16S rDNA sequence. The strain *Bacillus pumilus* INR-7 (16S rDNA partial sequence available in GenBank, accession number EU447665), which is commercialized in the U.S., was included in the study as an out-group. All the strains were stored at -80°C in TSB + 20% glycerol. Cultures were grown in LB medium for 24 h, and total DNA was extracted with the DNeasy® Blood and Tissue Kit (QIAGEN, Valencia

CA, 91355) with the addition of pretreatment of bacterial cells with 20 mg/ml lysozyme (Sigma L-6878, St. Louis MO, 63118) according to the manufacturer's instructions. Data for other bacilli strains were obtained from the GenBank (NCBI website) and all the details and accession numbers are presented in Table 3.2.

2.2. *PhyC* gene amplification

All the bacilli-PGPR strains were screened for the presence of the *phyC* gene using the primers FAR22 and FAR06 (see list of primers in Table 3.3). PCR was conducted with following conditions: denaturation at 95 °C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 8 min; and a final extension at 72°C for 2 min. The expected fragment size for this amplicon was 1.1 kb. Primers to amplify gen promoter (PP-01F/PP-01R) and terminator (PE-01F/PE-01R) were designed with the Primer3 software (Rozen and Skaletsky, 2000) based on the whole *phyC* sequence for the strains *B. amyloliquefaciens* FZB42 (Chen et al., 2007b), FZB45 (Idriss et al., 2002), and DS11 (Kim et al., 1998b) and on the partial sequence obtained in the present study for the strains FZB24, GB03, and MBI600. PP-01F primer anneals to the nucleotide positions -210 to -191 from the translation start of the FZB42 *phyC*, while PP-01R primer is specific to the nucleotide positions +204 to +224. PCR for promoter amplification included: denaturation at 95°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 45 sec, 72°C for 2 min; and a final extension at 72°C for 8 min. The expected amplicon size for this reaction was 435 bp. PE-01F primer was designed to anneal to the nucleotide positions +945 to +964 from the translation start of the FZB42 *phyC*, and PE-01R primer to the nucleotide positions +1307 to +1326. PCR was conducted with the same conditions and annealing temperature of the promoter amplification.

The expected amplicon size for the terminator primer set was 383 bp. For all PCR described above, GoTaq® DNA polymerase (Promega Corporation, Madison WI, 53711) was used according to manufacturer's instructions.

2.3. Amplification of genes for taxonomic analysis

Taxonomic classification of the bacilli-PGPR strains was based on the partial sequence of the 16S rDNA, *gyrA*, and *cheA* genes. Primers used for the genes are listed in Table 3.3. PCR conditions for 16S rDNA comprised: denaturation at 95°C for 5 min; 31 cycles of 94°C for 1 min, 57°C for 45 sec, 70°C for 2 min; and a final extension at 70°C for 8 min. Amplification of *gyrA* was performed as follows: denaturation at 95°C for 5 min; 31 cycles of 94°C for 1 min, –50°C for 45 sec, 72°C for 2 min; and a final extension at 72°C for 8 min. For *cheA*, PCR was performed as described for *gyrA*, but the annealing temperature was 52°C. In all the cases, GoTaq® DNA polymerase (Promega Corporation, Madison WI, 53711) was used according to manufacturer's instructions

2.4. DNA sequence analysis

PCR products were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia CA, 91355). Sequencing was conducted in the Genomics and Sequencing Laboratory at Auburn University using the same primers described for amplification and the consensus sequences were determined. DNA sequences were manually edited and alignment was done with Clustal X version 2.0 (Larkin et al., 2007). Phylogenetic analysis was performed using MEGA 4.0 with

default settings, using the Neighbor-joining algorithm and the bootstrap test with 1,000 samplings (Tamura et al., 2007). Open reading frames (ORF) were recognized using the ORfinder software (http://www.ncbi.nlm.nih.gov/projects/gorf/).

2.5. Determination of phytase production

Phytase production was determined by evaluating the capacity of the strains to grow on a defined medium using phytate as the only source of phosphorus (P). The defined medium provided the same mineral concentrations as the Spizizen's minimal medium (SMM), which supports good growth for most bacilli species (Harwood and Cutting, 1990), but included phytate as the only source of P and $CaCl_2$ as a source of Ca^{+2}, a cation required for the activity of *Bacillus* phytases (Kerovuo et al., 2000a; Oh et al., 2001). This medium contained (per liter): $(NH_4)_2SO_4$, 2 g; KCl, 15 g; Na_3 citrate•$2H_2O$, 1 g; $MgSO_4$•$7H_2O$, 0.2 g; $CaCl_2$•$2H_2O$, 0.294 g; and bacto agar (Difco), 15g. After sterilization in autoclave for 25 min, 10 ml of a solution 50% (w/v) of D-glucose, 10 ml of a solution 5 mg/ml of L-tryptophan, and 20 ml of a solution 100 mM of phytic acid sodium salt hydrate from rice (Sigma P0109, St. Louis MO, 63118), all filter sterilized, were added per liter of medium. As a negative control, all the strains were also plated on the same defined medium but with no source of P. Plates were incubated at 37 °C for 48 h and those strains growing clearly better on the medium with phytate were considered positive for phytase production.

Table 3.1 Selected *B. subtilis/amyloliquefaciens* PGPR strains screened for the presence of *phyC* gene

Strain and Current Taxonomic Classification	Commercial Product	Beneficial Effect	Amplification with FAR06 FAR22 primers[e]	Phytase activity[f]
Bacillus pumilus INR-7[a]	YieldShield®	PGP[d] and biocontrol	-	0
Bacillus amyloliquefaciens IN937a[b]	BioYield®	PGP and biocontrol	-	2
Bacillus subtilis GB03[c]	BioYield®	PGP and biocontrol	YES	3
Bacillus subtilis MBI600[c]	Subtilex®	Increased nodulation in legumes and biocontrol	YES	2
Bacillus amyloliquefaciens FZB24	RhizoPlus® FZB24® (WG, fl., and TB)	PGP and biocontrol	YES	3
Bacillus amyloliquefaciens FZB42	RhizoVital®42 fl.	PGP and biocontrol	YES	3
Bacillus amyloliquefaciens FZB45	Non commercialized	PGP	YES	3
Bacillus subtilis AP52[c]	Non commercialized	PGP and biocontrol	YES	3
Bacillus amyloliquefaciens AP136	Non commercialized	PGP and biocontrol	YES	3
Bacillus amyloliquefaciens AP188	Non commercialized	PGP and biocontrol	YES	2
Bacillus subtilis AP209	Non commercialized	PGP and biocontrol	-	1
Bacillus amyloliquefaciens AP218	Non commercialized	PGP and biocontrol	YES	3
Bacillus amyloliquefaciens AP219	Non commercialized	PGP and biocontrol	YES	3
Bacillus subtilis AP279	Non commercialized	PGP and biocontrol	-	1
Bacillus amyloliquefaciens AP295	Non commercialized	PGP and biocontrol	YES	3
Bacillus subtilis H57[c]	Non commercialized	PGP and biocontrol	YES	3
Bacillus subtilis UA321	Non commercialized	PGP and biocontrol	-	0
Bacillus subtilis UA322	Non commercialized	PGP and biocontrol	-	2

[a]Included as an outgroup.
[b]Classified later in this study as *B. subtilis*.
[c]Classified later in this study as *B. amyloliquefaciens*.
[d]PGP = Plant growth promotion.
[e]FAR22 and FAR06 are specific for *phyC* (Idriss et al., 2002).
[f]Phytase activity was determined by the capacity to grow on defined medium with phytic acid as the only P source. Growth is classified as 0= No growth, 1= poor, 2= good, and 3= abundant.

Table 3.2 List of sequences from the GenBank (NCBI website) included in this study

Strain and classification as in the NCBI web site	Accession number in GenBank				BLAST FAR22-FAR06 primers[b]	Phytase producer[c]	Report as a plant growth promoter[d]
	16S rDNA	*gyrA*	*cheA*	Phytase-coding sequence[a]			
B. amyloliquefaciens FZB42	AY055221	CP000560 7081-9462	CP000560 1627928-1629940	CP000560 2076283-2077984	YES	YES	YES
B. amyloliquefaciens FZB45	AY055224			AY055220	YES	YES	YES
B. subtilis/amyloliquefaciens B9601-Y2	FN652005	FN652790	FN652804	EU624118	YES	Not published	YES
B. subtilis E20	EU722405			FJ541287	YES	YES	NO
B. subtilis US417	AM501549			AM501550	YES	YES	NO
B. subtilis IDCC 1102	AY995569			DQ346197	YES	Not published	NO
B. subtilis IDCC 1103	AY995570			DQ346196	NO	Not published	NO
B. subtilis subsp. *subtilis* 168	AL009126 3177086-3178640	AL009126 6994-9459	AL009126 1712815-1714833	AL009126 2149808-2151506	NO	NO	NO
B. subtilis subsp. *spizizenii* ATCC 6633	NZ_ADGS01000029		AY212966	NZ_ADGS01000001 17907-19605	NO	Not published	NO
B. subtilis subsp. natto BEST195	AP011541 9755-11292	AP011541 6994-9459	AP011541 1720994-1723012	AP011541 2086856-2088554	NO	Not published	NO
B. amyloliquefaciens FZB24	AY055219					YES	YES
B. amyloliquefaciens ATCC 15841	AY055226					YES	NO
B. amyloliquefaciens UCMB-5017	AY211472	AY212970	AY212954			Not published	NO
B. amyloliquefaciens UCMB-5033	AY211473	AY212973	AY212957			Not published	NO
B. subtilis FZB37	AY055222					NO	NO
B. amyloliquefaciens DSM 7 (T)	AY055225	AF272015	AY212964			YES	NO
B. atrophaeus DSM 7264 (T)	AB021181	AF272016				Not published	NO
B. mojavensis DSM 9205 (T)	AB021191	AY212986	AY212965			Not published	NO
B. vallismortis DSM 11031 (T)	AB021198	AF272025				Not published	NO
B. subtilis subsp. *subtilis* DSM 10 (T)	AJ276351	AF272021				YES[e]	NO
B. subtilis subsp. *spizizenii* DSM 15029 (T)	AF074970	AF272020				Not published	NO

(T) = Type strain.
[a]Only phytase-coding sequences of strains for which 16S rDNA sequences were also available were included.
[b]FAR22-FAR06 primers are specific for *phyC* of *B. amyloliquefaciens* FZB45 (Idriss et al., 2002).
[c,d]References are through the text.
[e]Low production (Tzvetkov and Liebl, 2008).

Table 3.3 Primers used in this study

Primer	Sequence (5' → 3')	Target	Reference
FAR22	ATACTAGTCATATGAATCATTCAAAAACACTTTG	*PhyC* internal sequence	Idriss et al. (2002)
FAR06	AAGGATCCTTATTTCCGCTTCTGTCGG	*PhyC* internal sequence	Idriss et al. (2002)
PP-01F	TCTGATGCGCTTTCATATCG	*PhyC* promoter	This study
PP-01R	TTGGTTGTGATCAATTTGCTG	*PhyC* promoter	This study
PE-01F	AATTGACGTTCTGGGTTTCG	*PhyC* terminator	This study
PE-01R	ATATGACAAACCCGGGTGAA	*PhyC* terminator	This study
8F	AGAGTTTGATCCTGGCTCAG	16S rDNA	Edwards et al. (1989)
907R	CCGTCAATTCCTTTGAGTTT	16S rDNA	Lane et al. (1985)
CheA_MF	GAAACGGAKAYATGGMAGTBACMTCARACTGGCTG	*cheA*	Reva et al. (2004)
CheA_MR	TGCTCRAGACGCCCGCGGWCAATGACAAGCTCTTC	*cheA*	Reva et al. (2004)
GyrA_F	CAGTCAGGAAATGCGTACGTCCTT	*gyrA*	Reva et al. (2004)
GyrA_R	CAAGGTAATGCTCCAGGCATTGCT	*gyrA*	Reva et al. (2004)

3. Results

3.1. Screening for phytase production and presence of *phyC* gene

A PCR amplicon of the expected size was identified from 12 of the 18 bacilli strains tested (Table 3.1). All these positive strains also produced amplicons of the expected sizes when primers PP0-1F/PP-01R and PE-01F/PE-01R were used. In addition, these same strains grew well on defined medium with phytic acid as the only source of P, most of them displaying abundant growth (Table 3.1). In contrast, 4 of the 6 strains that did not produce amplicons showed a poor or null growth on the medium. From the GenBank search, 10 accession numbers were found for sequences of phytases from *Bacillus* strains for which the 16S rDNA sequences were also available (Table 3.2). Of those 10 phytase-coding sequences found in GenBank, 6

showed complementarity to the FAR22/FAR06 primers. Two of these complementary sequences corresponded to the strains *B. amyloliquefaciens* FZB42 and FZB45, which were also included in our previous screenings. The other 4 complementary sequences included 3 strains designated as *B. subtilis* (E20, US417, and IDCC 1102) and 1 strain (B9601-Y2) ambiguously designated (*B. subtilis* in some accessions and *B. amyloliquefaciens* in others).

3.2. Sequence analyses and taxonomic classification

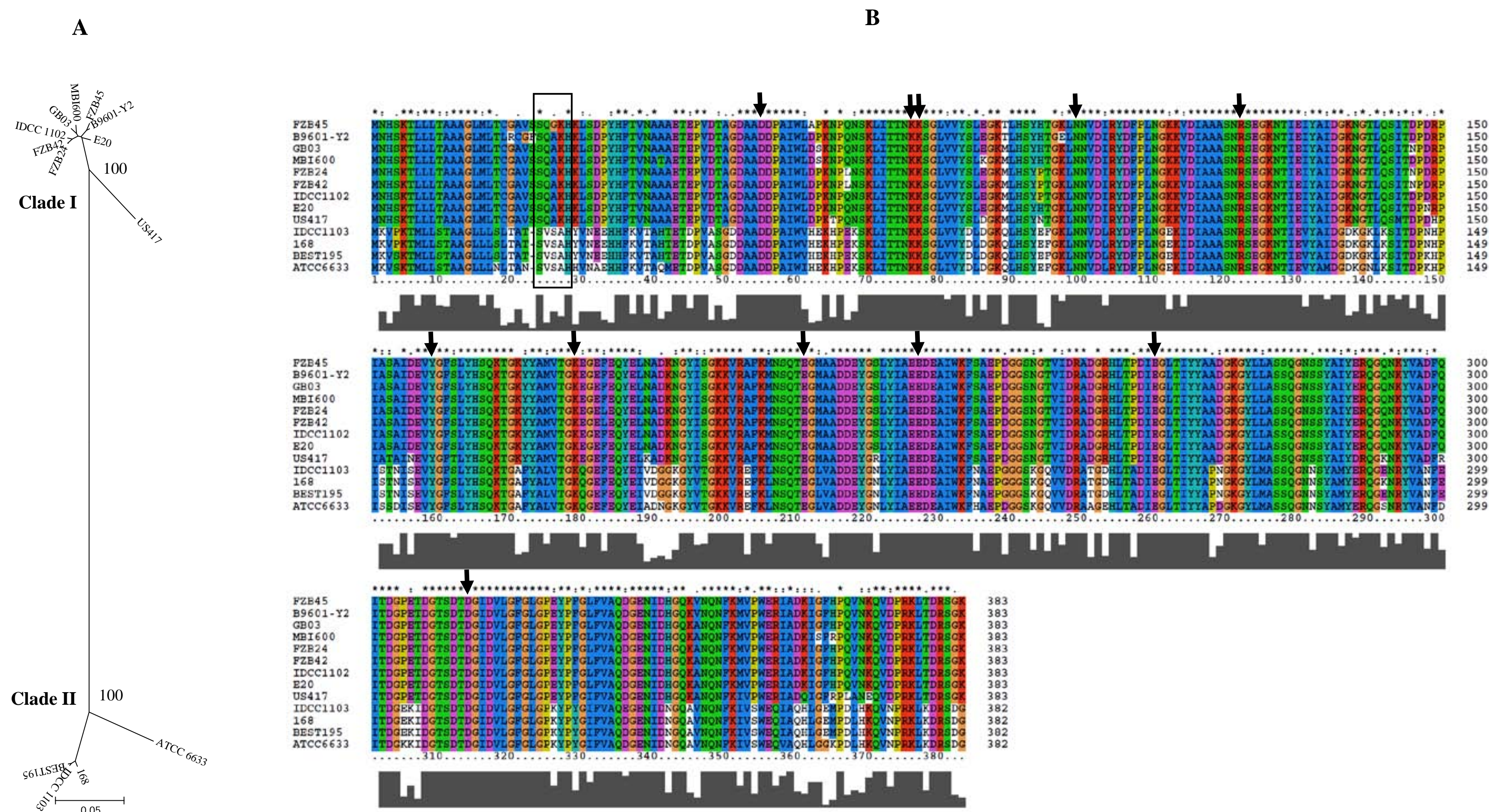
The predicted amino acid sequences for all 10 phytase-coding genes obtained from GenBank and for those obtained for the strains FZB24, GB03, and MBI600 were compared (Figure 3.1). This comparison showed two clearly separate clades of phytases with more than 96% similarity within each group (Figure 3.1A). Sequences that matched to the primers FAR22/FAR06 in the BLAST search grouped together (hereafter denominated clade I phytases) and were separated from the other 4 sequences that did not match (clade II phytases). Likewise, predicted amino acid sequences for strains FZB24, GB03, and MBI600 also grouped with the clade I phytases. Alignment of the predicted amino acid sequences revealed that all the 11 amino acid residues that were identified by Kerovuo et al. (2000b) as being part of the enzyme active site were conserved in all *Bacillus* phytases (Figure 3.1B). In contrast, the signal peptide cleavage site of the immature enzyme (SQA-KH) (Kerovuo et al., 1998; Nielsen et al., 1997) was only conserved in those phytases grouped in the clade I.

Further differences were found when comparing the promoter sequences of the phytase-coding genes. This analysis included 5 strains producing clade I phytases and 3 with clade II

phytases. Again, the same two clades of phytases were clearly separated based on the differences in the sequence of the phytase gene promoters (Figure 3.2A). Key sequences for interaction with RNA polymerase in FZB45 (Makarewicz et al., 2006) were well conserved across the *B. amyloliquefaciens* strains, while the *B. subtilis* strains displayed marked differences, especially in the -35 sequence and the additional PhoP binding box (Figure 3.2B).

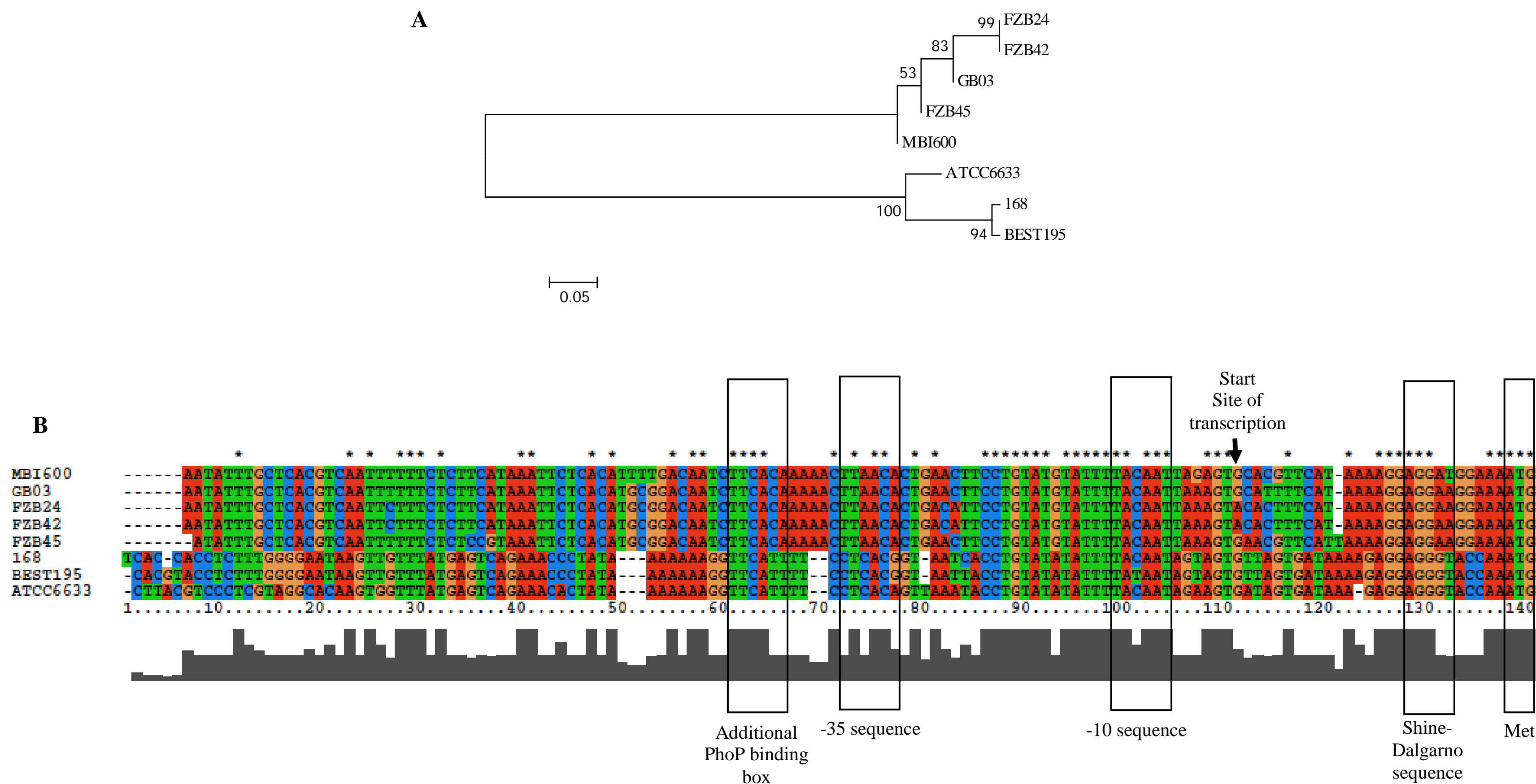
Comparison of the 16S rDNA sequences showed that all 4 strains with a predicted clade II phytase grouped together in a separate branch, along with the type strains *B. subtilis* subsp. *subtilis* DSM10 and *B. subtilis* subsp. *spizizenii* DSM 15029 (Figure 3.3A). In contrast, 16S rDNA sequences for 7 of the 8 strains with a clade I phytase were closely related to those of the type strain *B. amyloliquefaciens* DSM7 and the well-classified strains *B. amyloliquefaciens* FZB24, FZB42, FZB45, UCMB-5017, and UCMB-5033. Consistently, all those strains that were PCR positive for the presence of a clade I phytase also grouped with *B. amyloliquefaciens* strains. The 16S rDNA sequence of the strain B9601-Y2 that also possesses a clade I phytase was located in a separate branch and closer to the type strains *B. atropaeus* DSM 7264 and *B. vallismortis* DSM 11031. All these results were confirmed by the phylogenetic analysis based on the sequences of the genes *gyrA* and *cheA*, and in this case, strain B9601-Y2 was found to be more closely related to *B. amyloliquefaciens* strains (Figures 3.3B and 3.3C).

Figure 3.1 Comparison of the predicted-amino acid sequence for the phytase-coding genes present in several *B. subtilis/amyloliquefaciens* strains. A) phylogenetic tree and B) sequence alignment showing the signal peptide cleavage site (box) and the amino acids that take part in the active site (arrows)



Numbers at nodes in Figure 3.1A indicate bootstrap support values (1,000 samplings) for neighbor-joining analysis. In Figure 3.1B, the box indicates the signal peptide cleavage site (SQA-KH) according to Nielsen et al. (1997) and Kerovuo et al. (1998), and the arrows indicate the amino acid positions 55, 76, 77, 99, 122, 159, 179, 211, 227, 260, and 314, which take part in the enzyme active site and correspond to the positions 26, 47, 48, 70, 93, 130, 150, 182, 198, 231, and 285 of the mature protein (Kerovuo et al., 2000b).

Figure 3.2 Comparison of promoters of several phytase-coding genes. A) phylogenetic tree, B) sequence alignment with pointed sequences corresponding to the regions identified in *B. amyloliquefaciens* FZB45 as interacting with RNA polymerase (Makarewicz et al., 2006)



Phytases produced by strains MBI600, GB03, FZB24, FZB42, and FZB45 grouped in clade I., while phytases from strains 168, BEST195, and ATCC6633 corresponded to clade II. Numbers at nodes in Figure 3.2A indicate bootstrap support values (1,000 samplings) for neighbor-joining analysis.

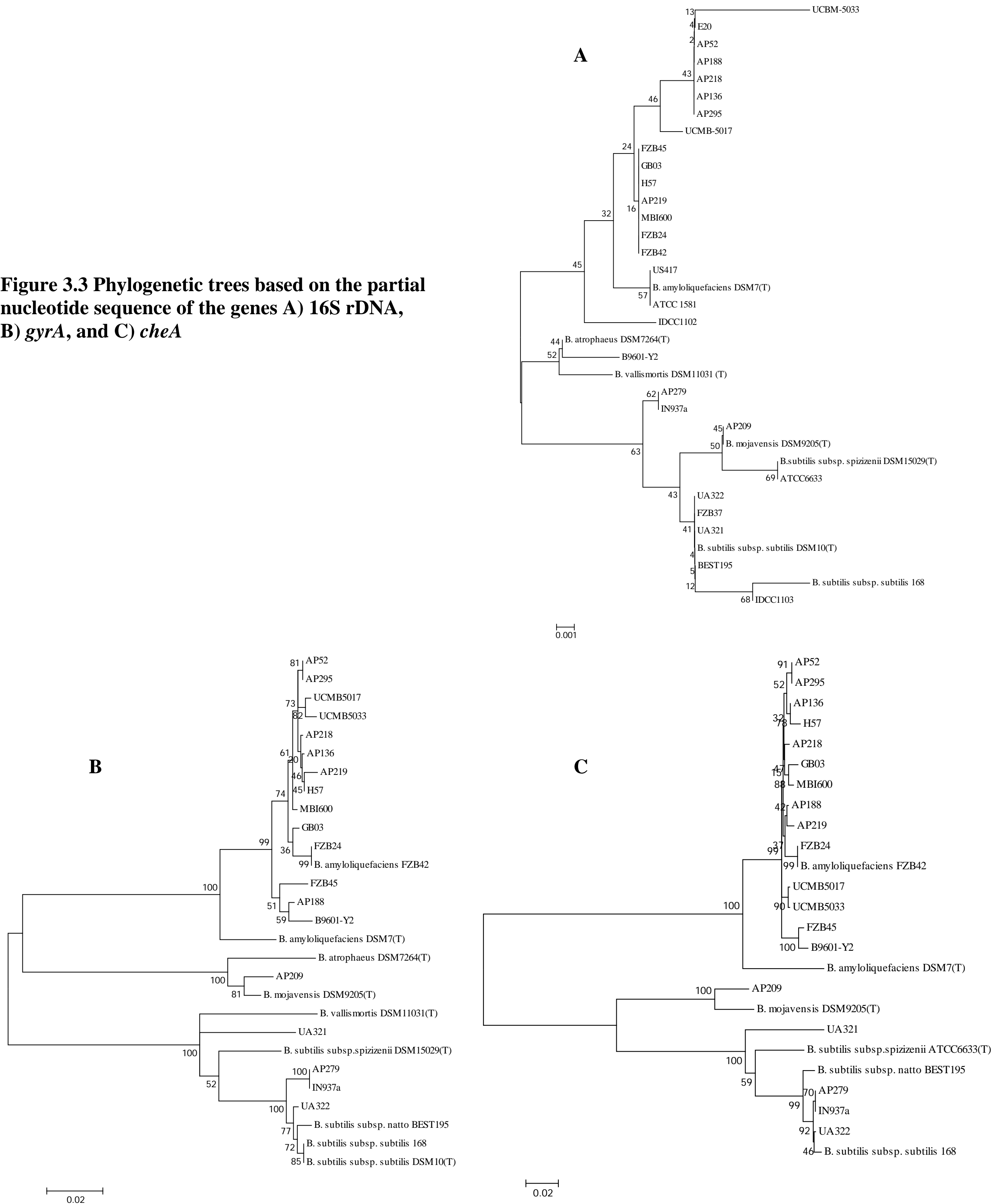


Figure 3.3 Phylogenetic trees based on the partial nucleotide sequence of the genes A) 16S rDNA, B) *gyrA*, and C) *cheA*

Sequences included in the analyses were obtained either by direct sequencing in the present study or from the GenBank (Table 3.2). Numbers at nodes indicate bootstrap support values (1,000 samplings) for neighbor-joining analysis.

Finally, 5 nucleotide positions in the 16S rDNA that were previously identified by Idriss et al. (2002) and Reva et al. (2004) as distinct in *B. subtilis* and *B. amyloliquefaciens* were evaluated in all the sequences included in this study. Our results show that, although all of the 5 positions tend to correlate with species discrimination, only the nucleotide positions 465, 472, and 483 were consistently different between the two species and conserved within the same species (Table 3.4). According to the secondary structure of the *B. subtilis* 16S rRNA described by Konings and Gutell (1995), these 3 nucleotides positions are located in the same arm and 2 of them correspond to a compensatory base change (Figure 3.4).

4. Discussion

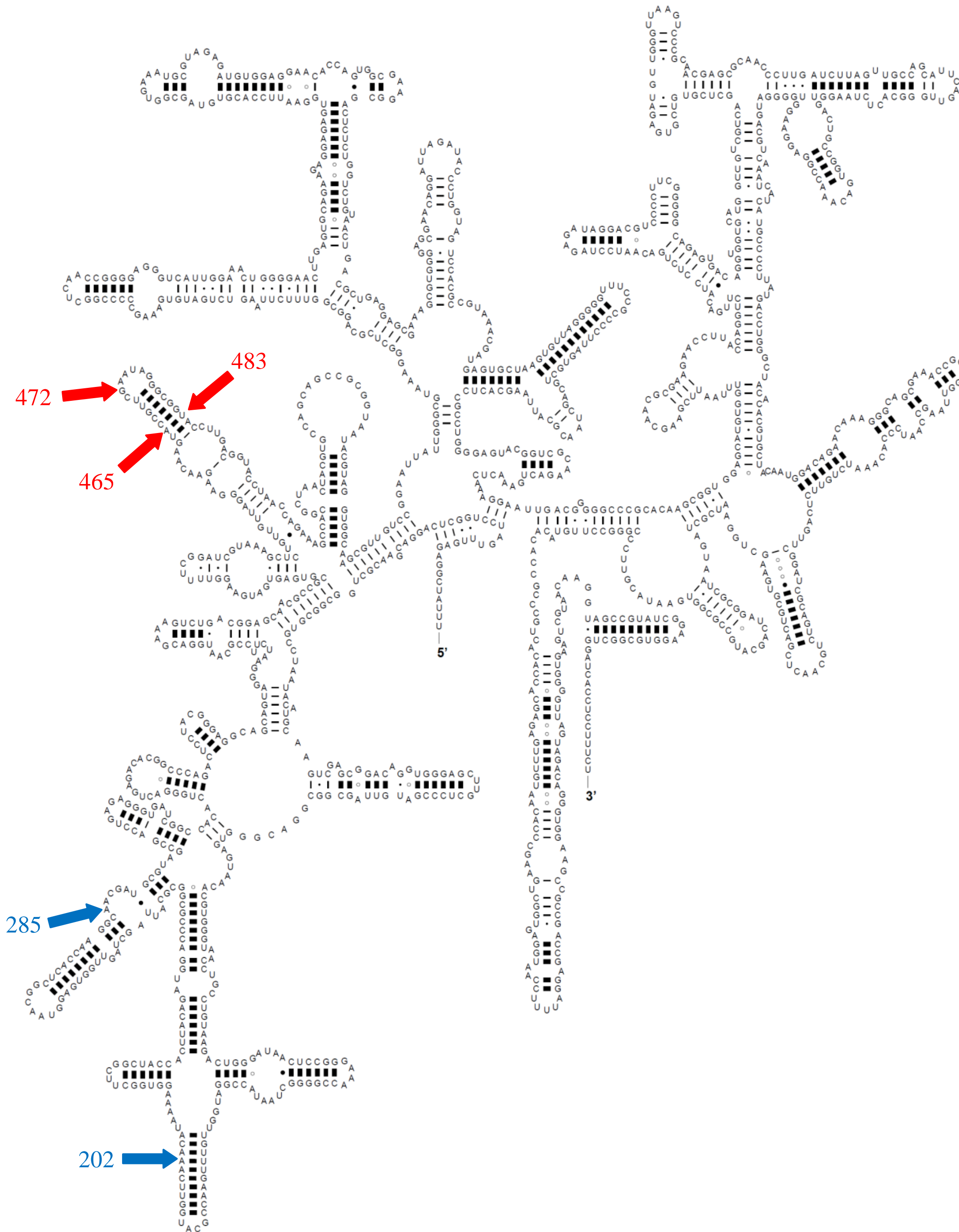
Our data suggest that *phyC* gene is well conserved across *B. amyloliquefaciens* strains, so it may be used as a genetic marker for quick differentiation between *B. subtilis* and *B. amyloliquefaciens* species. Clade I phytases were consistently present in those strains classified as *B. amyloliquefaciens* and they were absent in those strains more closely related to *B. subtilis* (Figure 3.1 and 3.3, and Table 3.4). Discrimination between these two bacterial species is difficult by phenotipic traits (Priest et al., 1987) and is ambiguous by BLAST analysis of the 16S rDNA sequence, the two most common techniques used for routine identification. Actually, several of the strains included in this study were previously misclassified (Tables 3.1 and 3.4). So, the primer pairs FAR22/FAR06, PP-01F/PP-01R, or PE-01F/PE01R can be used to easily determine by PCR if the query strain belongs to *B. amyloliquefaciens*.

Table 3.4 Summary of results for *phyC* gene analysis and taxonomic classification

Strain	Closest relative in phylogenetic analyses[a]	Diagnostic bases in 16S rDNA					Phytase	
		202	285	465	472	483	Clade	Production
DSM 7 (T)	*B. amyloliquefaciens*	G	G	G	A	C	I	High
AP218	*B. amyloliquefaciens*	G	G	G	A	C	I	Medium
AP219	*B. amyloliquefaciens*	G	G	G	A	C	I	High
AP295	*B. amyloliquefaciens*	G	G	G	A	C	I	High
GB03	*B. amyloliquefaciens*	G	G	G	A	C	I	High
H57	*B. amyloliquefaciens*	G	G	G	A	C	I	High
US417	*B. amyloliquefaciens*	G	G	G	A	C	I	N.D.
MBI600	*B. amyloliquefaciens*	G	G	G	A	C	I	Medium
FZB24	*B. amyloliquefaciens*	G	G	G	A	C	I	High
FZB42	*B. amyloliquefaciens*	G	G	G	A	C	I	High
FZB45	*B. amyloliquefaciens*	G	G	G	A	C	I	High
E20	*B. amyloliquefaciens*	G	G	G	A	C	I	N.D.
IDCC1102	*B. amyloliquefaciens*	G	A	G	A	C	I	N.D.
B9601-Y2	*B. amyloliquefaciens*	A	A	G	A	C	I	N.D.
ATCC 1581	*B. amyloliquefaciens*	G	G	G	A	C	N.D.	High
UCBM-5017	*B. amyloliquefaciens*	A	G	G	A	C	N.D.	N.D.
UCBM-5033	*B. amyloliquefaciens*	G	G	G	A	C	N.D.	N.D.
DSM 10 (T)	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	N.D.	Low
168	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	II	No
FZB37	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	N.D.	No
AP279	*Bacillus subtilis* subsp. *subtilis*	A	G	A	G	T	PCR negative for Clade I	Low
UA322	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	PCR negative for Clade I	Medium
UA321	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	PCR negative for Clade I	No
IN937a	*Bacillus subtilis* subsp. *subtilis*	A	G	A	G	T	PCR negative for Clade I	Medium
BEST195	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	II	N.D.
IDCC1103	*Bacillus subtilis* subsp. *subtilis*	A	A	A	G	T	II	N.D.
NBRC101239(T)	*Bacillus subtilis* subsp. *spizizenii*	A	A	A	G	T	N.D.	N.D.
ATCC6633	*Bacillus subtilis* subsp. *spizizenii*	A	A	A	G	T	II	N.D.
209	*Bacillus mojavensis*	A	A	A	G	T	N.D.	No

(T) = Type strain.
[a]Based on the results for 16S rDNA, *gyrA,* and *cheA*.

Figure 3.4 Secondary structure of the *Bacillus subtilis* 16S rRNA (Konings and Gutell, 1995) showing diagnostic nucleotide differences with *B. amyloliquefaciens* strains. Red and blue arrows indicate the nucleotide positions identified by Idriss et al. (2002) and Reva et al. (2004) as diagnostic, but only those positions indicated with red arrows were consistently different in the *B. amyloliquefaciens* strains included in this study



We show that phytase production is a common trait among bacilli-PGPR strains. Presence of a phytase-coding sequence similar to that of *B. amyloliquefaciens* FZB45 (encoding what was called here a clade I phytase, Figure 3.1) was consistently present in the majority of the strains evaluated (Table 3.1). All those strains harboring a clade I *phyC* gene were taxonomically related to *B. amyloliquefaciens*, while all the strains with a different gene type were classified as *B. subtilis* (Figure 3.3). In addition, those strains of *B. subtilis* for which phytase-coding sequences were available in the GenBank had a predicted AA sequence that is different from that in the phytases produced by *B. amyloliquefaciens* strains (around 70%; Figure 3.1).

In addition to the difference in the ORFs, promoter sequences of the phytase-coding genes of *B. amyloliquefaciens* and *B. subtilis* were also different (Figure 3.2). The additional PhoP-binding box and the -35 sequence identified by Makarewicz et al. (2006) displayed the greatest differences, which could affect the gene expression and explain the low or null level of phytase production in some *B. subtilis* strains (Idriss et al., 2002; Tzvetkov and Liebl, 2008). Likewise, the Shine-Dalgarno sequences also had some differences, suggesting that, in *B. subtilis* strains, not only reduced transcription could be occurring but also reduced translation. This would also be consistent with the fact that MBI600, which had 1 nucleotide different in its Shine-Dalgarno sequence, displayed a lower capacity to grow on defined medium with phytic acid as source on P. Nonetheless, transcription of *phyC* is complexly regulated. This gene even has a second control mechanism exerted by a global transient-phase regulator protein (Makarewicz et al., 2008), and thus, its expression could be affected in different steps.

Our results suggest that phytase production is distinct in strains of *B. subtilis* and *B. amyloliquefaciens*. Having in mind the importance of soil phytate as a source of P (Turner et al., 2002) and the natural low level of P in the rhizosphere (Hinsinger et al., 2005), those differences in phytase production could produce significant differences in phytate use and ecological fitness. Interestingly, experiments conducted with *Brassica* napus, *Hordeum* vulgare, and *Arabidopsis thaliana* showed that *B. amyloliquefaciens* strains were generally better root colonizers than members of the *B. subtilis* group (Reva et al., 2004). This different fitness for root colonization could be related to many different factors. For instance, production of several antibiotics is different between strains of those two species which could have an impact on the capacity of each species to compete in a highly competitive environment like the rhizosphere (Chen et al., 2009a). Nonetheless, an effect of different phytase production capacities on the fitness of both *Bacillus* species is also possible and should be tested in future studies.

Substrate utilization and ecological fitness have been proposed as the major drivers of bacterial speciation (Godreuil et al., 2005). Bacterial species have traditionally been defined according to arbitrary criteria such as DNA-DNA hybridization experiments and phenotypic characteristics (Stackebrandt and Goebel, 1994). This does not take into account the universal properties that define a species: 1) divergence is capped by a force of cohesion, 2) divergence between different species is irreversible, and 3) different species are ecologically distinct; properties that are held by ecotypes (Cohan, 2002). Therefore, changes in some bacterial ecotypes leading to improved fitness, *e.g.* better substrate utilization, can produce new ecotypes and drive speciation (Cohan, 2006). This has been supported by studies with strains of the *Bacillus subtilis-Bacillus licheniformis* clade from sites differing in solar exposure and soil

texture (Connor et al., 2010). Here, ecotypes demarcated by computer simulation were found to be significantly different in their association to the environmental factors evaluated and nearly identical to the recognized species and subspecies of the *Bacillus subtilis-Bacillus licheniformis* clade.

Results obtained here show that 16S rDNA sequencing could still be useful to differentiate between *B. subtilis* and *B. amyloliquefaciens*, despite the high similarity of this gene in these two species. Sequence similarity for the 16S rDNA of the two species is greater than the 97% traditionally accepted (Stackebrandt and Goebel, 1994) and even than the 98.7-99% recently proposed (Stackebrandt and Ebers, 2006) for reliable identification of bacterial strains to the species level. However, nucleotides at positions 465, 472, and 483 may serve as diagnostics for identification (Table 3.4 and Figure 3.4). The paired nucleotides 462-483 are particularly interesting as they represent a compensatory base change (CBC) that seems to be well fixed in the molecule across the population. In the rDNA sequence ITS2 of eukaryotes, the presence of only 1 CBC appears to be sufficient for discrimination of species (Müller et al., 2007). There is no clear evidence yet indicating that this can also be the case in prokaryotes; however, this could be an interesting topic to research and it could have important implications for bacterial classification.

Future studies with a larger database will establish the extent of the discrimination among *B. subtilis* and *B. amyloliquefaciens* strains that may be achieved through molecular analysis of phytase gene sequences. We expect that in addition to its phylogenetic value, the phytase

molecular analysis will contribute to understanding the molecular bases of bacilli ecology and the potential of *B. subtilis/amyloliquefaciens* strains as PGPR.

Chapter 4. Bacilli PGPR Induce Changes in Root Architecture and Alter Phosphorus Uptake During Early Growth of Chinese Cabbage

1. Introduction

Plant root systems are essential for plant growth due to their role in water and nutrient uptake from soil and the architecture of root systems is as a key factor for plant nutrient uptake (Lynch, 1995). Nutrient distribution in soil is not homogeneous and specific patterns of accumulation and availability exist (Jobbágy and Jackson, 2001). Thus, root distribution in soil determines the plant's capacity to explore soil resources in an efficient way (Gregory, 2006; Thaler and Pagès, 1998). This aspect has been commonly overlooked in plant growth experiments which generally use homogeneous substrates (Hutchings and John, 2004). Breeding programs, in contrast, have frequently selected for traits related to advantageous root architecture (de Dorlodot et al., 2007). However, root architecture is not completely predetermined by the genotype, and plants have developed the capacity to adapt to nutrient availability by changing the architecture of their root system (López-Bucio et al., 2003). Those changes in root architecture are usually mediated by plant growth regulators such as cytokinins (Aloni et al., 2006), indole-acetic acid (IAA) (Gaspar et al., 2002), and ethylene (Rahman et al., 2002).

The most dramatic alteration of root architecture occurs in response to low soil phosphorus (P) (López-Bucio et al., 2003). This could be explained by the importance of P as a plant nutrient and its frequently limited soil availability under natural conditions (Vance et al., 2003). Phosphorus has a very low mobility in soil due to its precipitation as inorganic complexes with calcium, free oxides, or hydroxides of aluminum and iron, and to its adsorption to the surfaces of clays and organic matter (Fox, 1981; Raghothama and Karthikeyan, 2005). Root architecture changes driven by P-limiting conditions have mainly been characterized in the model plant *Arabidopsis thaliana*. Under low phosphate availability, *Arabidopsis* shows increased lateral root density and length, reduced primary root growth through reduced cell elongation (Williamson et al., 2001), and longer root hairs (Bates and Lynch, 1996). According to the experimental evidence, all these changes are mostly mediated by increases in the level and sensitivity to auxins (especially IAA) (Lopez-Bucio et al., 2002; Perez-Torres et al., 2008) and ethylene (Ma et al., 2003), and to a lesser extent, cytokinins (Aloni et al., 2006).

This response of *Arabidopsis* to low P availability favoring lateral root growth over primary root growth is viewed as an adaptative response to soil P distribution (Williamson et al., 2001). Almost invariably, the levels of P in soil are higher in the upper layers (Jobbágy and Jackson, 2001); therefore, shallower root systems are more efficient at exploring those layers and foraging for P (Walk et al., 2006). This hypothesis has been extensively supported by studies on common bean, a principal food in tropical and subtropical regions where soil P is limiting (Bonser et al., 1996). Common bean, like *Arabidopsis*, responds to P deficiency by increasing lateral root growth (Liao et al., 2001). In addition, some bean genotypes form shallower root systems, and this characteristic is highly correlated with better plant growth in P-limited soils. Studies with

computer models (Lynch and Brown, 2001) and experiments in the greenhouse and field (Rubio et al., 2003) indicated that root shallowness conferred a competitive advantage when phosphorus availability was concentrated in the topsoil but not when phosphorus availability was uniformly low or high throughout the soil layers

Some bacterial strains that are referred to as plant growth-promoting rhizobacteria (PGPR) also cause alterations of root architecture (Glick et al., 1999). These changes, depending on the PGPR strain and inoculum concentration, can include increases or reductions of primary root length (Patten and Glick, 2002; Persello-Cartieaux et al., 2001), stimulation of lateral root number and length (Larcher et al., 2003), and elongation of root hairs (Desbrosses et al., 2009). The mechanisms by which PGPR cause such changes are not clearly understood and evidence is contrasting. Nonetheless, important insights have been gained, suggesting a role for production or alteration of plant levels of IAA (Patten and Glick, 2002), ethylene (Li et al., 2000), and cytokinins (Ortíz-Castro et al., 2008). Additionally, production of volatile organic compounds (VOCs) has shown *in vitro* effects (Zhang et al., 2007). The most described system involves the stimulation of primary root length by the combined action of IAA and reduced levels of ethylene (a root-elongation inhibitor) which results by degradation of the ethylene precursor aminocyclopropane-1-carboxylate (ACC) by the bacterially-produced enzyme ACC-deaminase (Glick et al., 1998; Glick et al., 2007).

The effect of the interaction between root architecture changes induced by PGPR and soil nutrient content, as well as the effect of this interaction on plant nutrition, has not been extensively investigated. Interestingly, plant responses to PGPR inoculation such as increased

number and length of lateral roots, and root hair elongation resemble those of P deficiency (Williamson et al., 2001). This could suggest that root architecture changes induced by PGPR could have an effect on plant P uptake. In addition, PGPR inoculation will likely alter the ecology of the rhizosphere since the inoculated bacteria will use carbon substrates from the plant and other minerals present in this portion of the soil (Gregory, 2006). Such alteration of rhizosphere ecology should be particularly pronounced when bacilli PGPR are inoculated given the marked P depletion in the rhizosphere (Hinsinger et al., 2005) and the key role that is played by P in bacilli biology. Bacilli PGPR, also called aerobic endospore-forming bacteria (AEFB), represent most of the PGPR-based products due to their capacity to form highly resistant endospores, which are the bacterial structures inoculated in practice (Kloepper et al., 2004). In this bacterial group, besides all the regular metabolic functions, P is a critical element in the sporulation process which is triggered under P-limiting conditions (Sonenshein, 2000).

The present study aimed to characterize the effect of inoculation with bacilli-PGPR on root architecture of Chinese cabbage during early growth and to relate this to soil P content and plant uptake of P. For this purpose, a collection of 73 plant-associated bacilli strains from the Auburn University Phytobacteriology Laboratory were screened for production of IAA and ACC-deaminase. Then, four well-known bacilli-PGPR strains were tested for their effects on the root architecture of Chinese cabbage in a gnotobiotic system. Lastly, a soil-plant system was used to evaluate the effect of *Bacillus amyloliquefaciens* FZB42, a well-characterized PGPR strain (Chen et al., 2007b), on root architecture and P uptake of Chinese cabbage grown at different soil P levels.

2. Materials and Methods

2.1. Bacterial strains and inoculum preparation

Seventy-one strains of *Bacillus* spp. (bacilli) from the Phytobacteriology Laboratory at Auburn University (U.S.A.) and two (UA321 and UA322) from the Microbiological Control Laboratory at University of Antioquia (Colombia) were used for the screening study. This collection included seven different species according to identification by 16S rDNA sequence (Table 4.1). The sources of the strains are diverse and include some commercially available PGPR strains that belong to *Bacillus subtilis/amyloliquefaciens* group. All strains were stored in TSB + 20% glycerol at -80°C. For experiments where plants were inoculated, bacterial spore suspensions were always used. Those spore suspensions were prepared by growing the strain on a modified medium to stimulate sporulation. One liter of medium contained proteose peptone (vegetable, Fluka), 3.3 g; beef extract powder, 1.0 g; D-lactose monohydrate, 5.0 g; NaCl, 5.0 g; K_2HPO_4, 2.0 g; KCl, 1.0 g; $MgSO_4 \bullet 7H_2O$, 0.25 g; $MnSO_4$, 10 mg; and agar, 18 g. After incubation for 7 days at 28ºC, spores were harvested from medium surface using 5 ml of sterile distilled water (SDW) per Petri dish. The resulting suspension was centrifuged at 3,000 rev/min for 10 min; the supernatant was discarded; and the pellet was resuspended in SDW. This suspension was pasteurized for 15 min at 80ºC, and the concentration of spores was determined by plate counting. The spore suspension was stored at 4ºC until use, and the spore viability was confirmed at the moment of inoculation.

2.2. Determination of IAA-like compounds production

Production of IAA-like compounds by all the 73 strains was determined *in vitro* according to Patten and Glick (2002) with slight modifications. The strains were grown on TSA for 24 h at 26°C. Flasks of 125-ml containing 25 ml of TSB supplemented with 500 µg/ml tryptophan (FisherBiotech, BP395) were inoculated with each of the strains. Flasks were incubated at room temperature with 150 rev/min on an orbital shaker for 48 h. Afterwards,$O.D._{600}$ was recorded as an indicator of growth, and an aliquot of each flask was centrifuged (8000 rev/min) to remove bacterial cells. One ml of supernatant was mixed with 4 ml of Salkowski's reagent (150 ml of 18 M H_2SO_4, 250 ml distilled water, and 7.5 ml of 0.5 M $FeCl_3 \bullet 6H_2O$). Absorbance at 535 nm was measured after 20 min (Gordon and Weber, 1951; Patten and Glick, 2002). IAA concentration was estimated by comparison with a standard curve prepared with indole-3-acetic acid (Sigma I-2886). In a separate experiment, production of IAA-like compounds by *B. amyloliquefaciens* strain FZB42 under different nutritional conditions was tested. FZB42 was grown on TSA for 24 h and then transferred to LB medium. Twenty µL of a 24 h culture were transferred to 125-ml flasks containing 25 ml of 6 different media: 10% TSB, 10% TSB + tryptophan, full strength TSB, full strength TSB + tryptophan, LB, and LB + tryptophan. Samples were taken under aseptic conditions 24, 48, and 72 h after inoculation; $O.D._{600}$ and IAA were determined as described above.

2.3. Determination of ACC deaminase production

Determination of ACC deaminase production by 66 of the 73 strains was conducted by cultivation on a defined medium using ACC as the only source of nitrogen (Penrose and Glick, 2003); *B. mycoides* strains were not tested because of extremely poor growth on all the defined media evaluated in the standardization assays. All strains other that *B. mycoides* grew well on Spizizen's minimal medium (SMM) (Harwood and Cutting, 1990). SMM contained (per liter): $(NH4)_2SO_4$, 2g; K_2HPO_4, 14g; KH_2PO_4, 6g; Na citrate.$2H_2O$, 1g; $MgSO_4$•$7H_2O$, 0.2g; and bacto agar (Difco), 15g. After autoclaving for 25 min, 10 ml of a solution 50% (w/v) of D-glucose and 10 ml of a solution 5 mg/ml of L-tryptophan, both filter sterilized, were added per liter of medium. Two other variants of this medium were prepared: 1) by replacing $(NH_4)_2SO_4$ with 3.0 mM 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma A3903) and 2) by not adding any N source. Two ACC-positive pseudomonad strains that grew well on SMM medium were used as positive controls, and these strains grew well on SMM + ACC but not on SMM without any N source.

2.4. Determination of P solubilization

Phosphorus solubilization capacity was determined for all the 73 bacilli strains by growing the strains in 125-ml flasks containing 25 ml of liquid National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). Composition of this medium (per liter) was: glucose, 10 g; $Ca_3(PO)_4$, 5 g; $MgCl_2$•$6H_2O$, 5 g; $MgSO_4$•$7H_2O$, 0.25 g; KCl, 0.2 g' and $(NH_4)_2SO_4$, 0.1 g. Flasks were shaken at 150 rev/min for 48 h at room temperature, and then

cells were precipitated by centrifugation for 10 min at 3000 rev/min. P in solution was determined in the supernatant by the molybdate-blue method (Murphy and Riley, 1962).

2.5. Effect of bacilli PGPR inoculation on Chinese cabbage root architecture

Bacilli strains FZB42, GB03, and MBI600, which are part of commercial products available in the U.S., and the strain UA321 were tested for their effect on root architecture of Chinese cabbage in a gnotobiotic root pouch assay, following the method by Penrose and Glick (2003) with modifications. Seeds of Chinese cabbage (*Brassica rapa* Kaboko Hybrid, Park Seed Co., Greenwood, S.C. 29647) were disinfected by soaking in 70% ethanol for 1 min, rinsing once with SDW, then soaking again in 0.5% sodium hypochlorite for 10 min, and finally rinsing 15 times with SDW. Then, sterile seed germination pouches (Cyg, Mega International, St. Paul, MN, USA) were used, placing 5 seeds per pouch. Pouches were maintained in covered transparent plastic boxes at 25ºC in a growth chamber with 16 h light and 8 h darkness for 2 days. The seedlings were then inoculated with 100 µl of 10^7, 10^8, 10^9 spores/ml of each strain and with a water control. Five pouches were used for each treatment (25 seedlings total) and evaluation was conducted 5 days after inoculation, incubating under the same conditions mentioned above. For each plant, the main root length was recorded, and all the lateral roots were counted and classified into four length ranks: 0-0.5, 0.5-1.0, 1.0-1.5, and 1.5-2.0 cm. Comparison of the effect of each bacterial concentration was done comparing the means and the respective standard errors (SE).

2.6. Soil characterization

The soil used for soil-plant experiments was the surface horizon of a Marvyn loamy sand (fine-loam, kaolinitic, thermic Typic Kanhapludults) from the Cullars Rotation experiment, which is the oldest continuous soil fertility study in the southern U.S. This experiment is maintained by the Department of Agronomy and Soils at Auburn University, the Alabama Agricultural Experiment Station, and USDA-ARS-Soil Dynamics Laboratory. Soil was taken from plots that had not received any P fertilization since 1911. Chemical analysis was routinely done in the Soil Testing Laboratory at Auburn University using Mehlich-1 extract. Determination by inductively coupled argon plasma spectrophotometry (ICAP) showed the following parameters: pH 6.47, organic matter 0.6%, and extractable nutrient content (mg/kg soil): P, 4.1; K, 74.8; Mg, 24.9; Ca, 302.7; Al, 101; B, 0.4; Cu, 1.4; Fe, 3.6; Mn, 11.4; Na, 15.4; and Zn, 0.9. A mixture 1:1 (w/w) soil:sand was used for all the experiments. Mehlich-1 extractable P less than 6 mg/kg soil is considered very low by Auburn University Soil Testing Laboratory (Adams et al., 1994). A P-sorption curve establishing the relationship between P applied and P in soil solution was prepared for this mixture (Fox and Kamprath, 1970b; Nair et al., 1984). For this purpose, both 1.5 g of 4 mm-sieved, air-dried soil and 1.5 g of sand (3 g soil mixture total) were put into 50 ml polypropylene tubes. Thirty ml of 0.01 M $CaCl_2•2H_2O$ containing proper amounts of KH_2PO_4 were added into separate tubes in order to reach concentrations equivalent to 0, 5, 10, 15, 20, 25, and 30 mg P/kg soil. Two drops of chloroform were added into each of the tubes, which were then placed horizontally in an orbital shaker for 24 h at room temperature and 150 rev/min. After incubation, samples were centrifuged for 5 min

at 3000 rev/min and filtered through filter paper Whatman No. 1. Phosphorus in solution was determined using the molybdate-blue method (Murphy and Riley, 1962).

2.7. Effects of *B. amyloliquefaciens* FZB42 at different soil P levels

The effect of *Bacillus amyloliquefaciens* FZB42 on Chinese cabbage root architecture and P uptake at different soil P levels was tested. This strain, which has been used as a model in many past studies of bacilli PGPR (Chen et al., 2007b) was kindly provided by Dr. Rainer Borriss (Institut für Biologie, Humboldt Universität, Berlin). Seeds of Chinese cabbage were disinfected as described above for the root pouch assay. Then, they were transferred to Petri dishes with 2% water-agar and incubated for 2 days at 28ºC in the dark, until germination occurred. These seedlings were individually planted in plant growth containers which consisted of 50 ml-plastic centrifuge tubes containing 80 g of a mixture 1:1 (w/w) soil-sand. The amounts of soil (air-dried and sieved at < 4mm) and sand (40 g each) to be added into each container were weighed separately and then mixed to ensure homogeneity. These containers were fertilized individually with 14 ml (to reach 60% of soil maximum water-holding capacity, MWHC) of a solution containing (per liter): NH_4NO_3, 80 mg; KNO_3, 607 mg; $Ca(NO_3)_2 \bullet 4H_2O$, 2.834 g; $MgSO_4 \bullet 7H_2O$, 1.479 g; H_3BO_3, 8.58 mg; $MnCl_2.4H_2O$, 5.43 mg; $ZnSO_4$, 0.66 mg; $CuSO_4.5H_2O$, 0.24 mg; $H_2MoO_4.H_2O$, 0.06 mg; and EDTA-FeNa, 150 mg. This fertilization was equivalent to (mg/kg soil): N, 79; K, 41; Ca, 84; Mg, 26; B, 0.263; Mn, 0.264; Zn, 0.047; Cu, 0.011; Mo, 0.006; and Fe, 4.

The experiment was conducted as a 6 x 4 factorial arrangement, evaluating 6 levels of bacterial inoculation (5 spore concentrations and a non-inoculated control) at 4 different levels of soil P (homogeneous distribution of 0, 5, and 15 mg P/kg soil and one treatment with heterogeneous distribution of P with 15, 5, and 0 mg P/kg from top to bottom). Soil P treatments were applied by adding the corresponding amount of P to the nutrient solution. For this, nutrient solutions for treatments with 5 and 15 mg P/kg soil received 110.7 and 332.1 mg NaH_2PO_4/l, respectively. For treatment with heterogeneous distribution of P in soil, a gradient was achieved as follows. First, 27 g of soil:sand mix were poured into the container and fertilized with 4.725 ml of nutrient solution without P. Next, another 27 g of soil:sand mix were added and fertilized with 4.725 ml of nutrient solution containing 110.7 mg NaH_2PO_4/l. Lastly, 26 g of soil:sand mix were added and fertilized with 4.55 ml of nutrient solution containing 332.1 mg NaH_2PO_4/l. This resulted in containers with 15, 5, and 0 mg P/kg soil in the top third, middle third, and bottom third, respectively. FZB42 inoculation was conducted by applying 100 µl/seedling of 10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml and a non-inoculated control with sterile water. Soil moisture was maintained between 40-60% of MWHC, and plants were incubated at 25ºC in a growth chamber with 16 h light and 8 h darkness.

Ten containers, each having a 2-day old seedling, were used for each of the 24 combination treatments. Five containers were evaluated 7 days after inoculation (DAI) and the other 5 were evaluated 14 DAI. This experiment was conducted twice. The evaluation at 7 DAI included three root architecture variables: total root length, root surface area, and number of root tips. The evaluation at 14 DAI included total root length, root surface area, fresh shoot weight (FSW), and shoot P_i content. Root architecture parameters were determined by scanning the roots previously

stained with cotton blue for at least 5 min and images were analyzed by using WinRHIZO software (V5.0, Regent Instruments, Quebec, Canada).

For each evaluation time, response data from the two plant-soil experiments were analyzed jointly as a factorial with bacterial inoculation and P level as fixed effects. The effect for experiment was extracted from the residual error term and treated as a random effect. Normality and equal variances assumptions were first evaluated using the student panel graphs generated by SAS® GLIMMIX Procedure, which was also used for all the analyses. Normal distribution was warranted for total root length and root surface area at both 7 and 14 DAI; however, number of root tips, fresh shoot weight, and P_i content exhibited a log-normal distribution. As the equal variance assumption was not fulfilled for any of the response variables, the variance structures were modeled (*R*-side of the covariance parameters of SAS) using the group option to create homogeneous variance groups. A smaller AIC value from the 'information criteria' output and a better graphical residual distribution were considered as indicators of a good fit for the model. The residual term was the pooled residual within bacterial inoculation × P level combination variation, as the experimental design was a CRD. For response variables with a statistically significant interaction, bacterial concentration was compared to the non-inoculated control within each P level using Dunnett's test. In contrast, when only P level was significant, main effects were compared by using the simulate adjustment of GLIMMIX procedure for which significance classes are presented in the graphs. Pearson product-moment correlation coefficient between the least square means of shoot P_i content and fresh shoot weight was calculated using PROC CORR of SAS.

2.8. Plant P extraction and determination

Whole plant $P_i$was extracted following the method by Huang et al. (2005b). For this purpose, the shoot of each plant was washed in distilled water and put into P-free 20 ml-glass tubes containing 15 ml 0.1 M H_2SO_4. Samples were shaken end-to-end for 16 h at 25ºC, then heated in a water bath at 85ºC for 15 min, and cooled down for P_i determination. P in solution was determined in the extractant by the molybdate-blue method (Murphy and Riley, 1962) using a P standard curve also prepared in 0.1 M H_2SO_4. P_i was expressed as shoot P_i content (µg/shoot).

3. Results

3.1. Screening of plant-associated bacilli for *in vitro* production of ACC deaminase and IAA, and P solubilization

Results of the screening of plant-associated bacilli for in vitro production of ACC deaminase, IAA, and P solubilization are shown in Table 4.1. None of the strains grew on defined SMM medium using ACC as the only N source, indicating that no strain produced ACC deaminase. In contrast, 68% of the strains produced IAA-like compounds. Strains that produced IAA-like compound were more common in the species *B. amyloliquefaciens*, *B. megaterium*, and *B. simplex*, with the last two species containing the largest numbers of producing strains.

In vitro P solubilization of $Ca_3(PO)_4$ was common among the strains tested with 85% of the strains displaying this trait. All the strains belonging to *B. amyloliquefaciens*, *B. megaterium*, and

B. simplex solubilized P *in vitro*. *B. mycoides*, in contrast, was the species with the lowest percentage of *in vitro* P solubilizers.

Table 4.1 *In vitro* P solubilization and production of ACC deaminase and IAA- like compounds in a collection of plant-associated bacilli of the Auburn University phytobacteriology laboratory

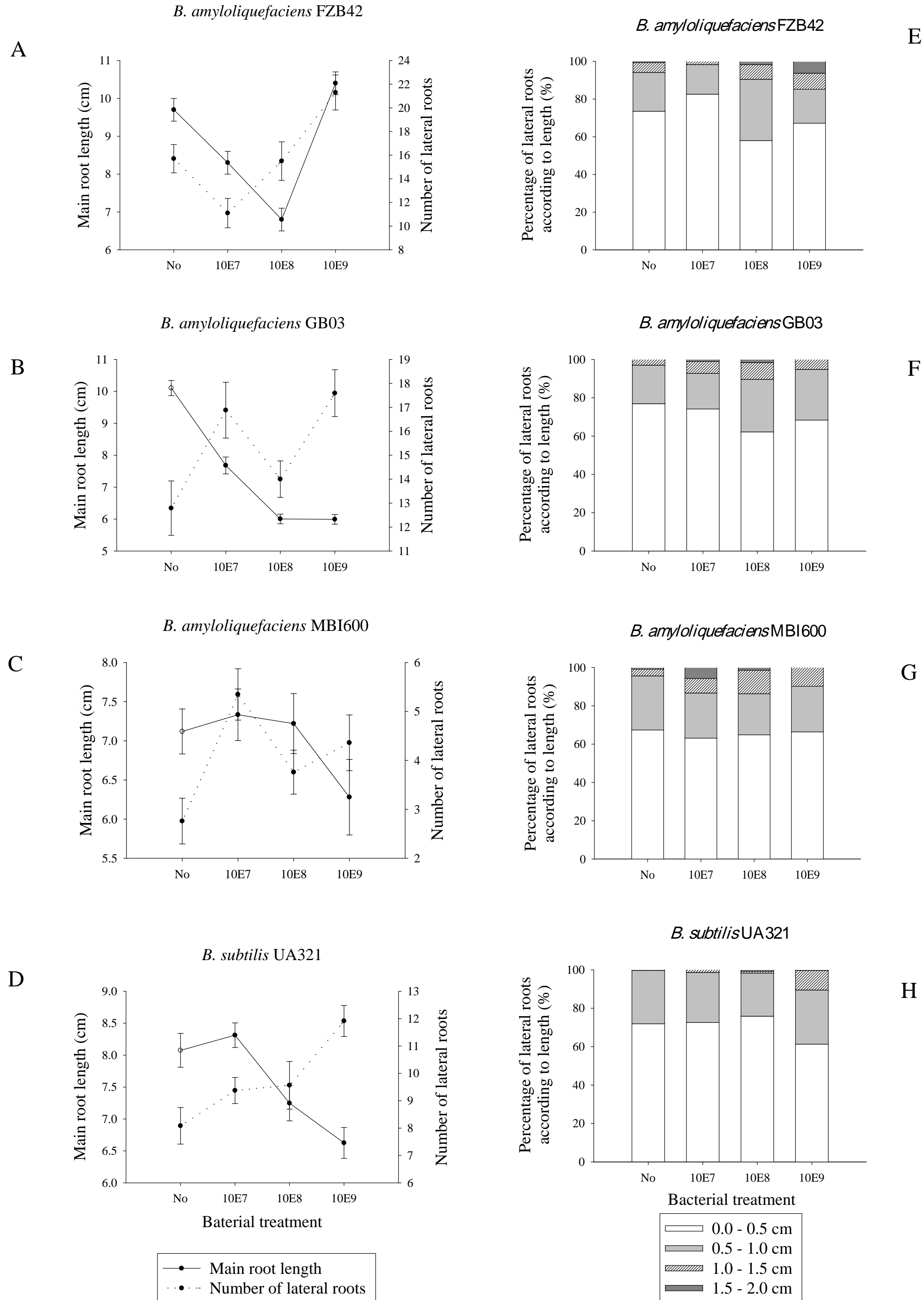
Species	Number of strains tested	ACC deaminase Strains positive	IAA-like compounds		P solubilization	
			Strains positive	Range of production (µg IAA/ml /OD_{600})	Strains positive	Range of P solubilized (mg/l)
B. amyloliquefaciens	12	0	10 (83%)	0.2 - 2.6	12 (100%)	5.1 - 10.0
B. subtilis	4	0	2 (50%)	0.4	3 (100%)	5.5 - 10.4
B. megaterium	8	0	7 (88%)	3.3 - 8.5	8 (100%)	5.3 - 22.6
B. simplex	35	0	26 (74%)	0.1 - 10.7	32 (91%)	0.5 - 22.1
B. mycoides	7	NT	4 (57%)	0.2 - 1.4	3 (43%)	0.3 - 3.6
B. pumilus	2	0	1 (50%)	2.7	1 (50%)	6.5
B. safensis	5	0	0 (0%)	-	3 (60%)	0.4 - 6.9
Total	**73**	**0**	**50 (68%)**		**62 (85%)**	

Bacterial species correspond to the identification by 16S rDNA sequence. ACC deaminase was determined by growth on a defined medium (SMM) using ACC as the only source of N and it included well-known ACC deaminase-producing strains as positive controls. NT representstrains not tested because of poor growth on SMM medium with $(NH4)_2SO_4$. IAA production was determined colorimetrically with Salkowski solution in the supernatant of 48 h–old liquid cultures in TSB + 500 µg tryptophan /ml. P solubilization was determined by growing the strains for 48 h in liquid culture containing $Ca_3(PO_3)_2$ as the only source of P.

3.2. Effect of bacilli PGPR inoculation on Chinese cabbage root architecture

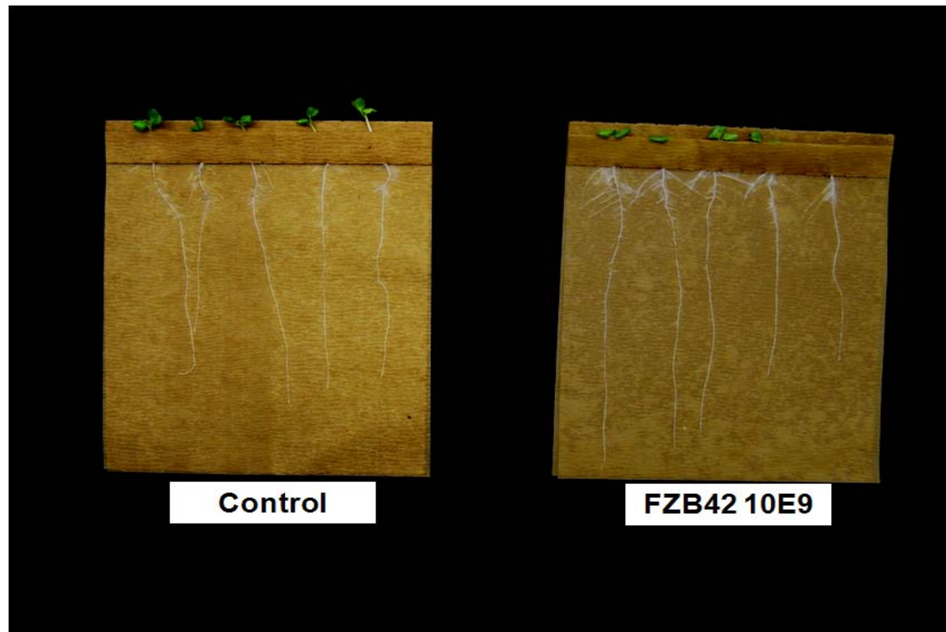
All of the tested bacilli PGPR strains caused significant changes in the root architecture of Chinese cabbage 5 days after inoculation at all three concentrations tested. As the inoculum concentration increased, the general trend of root response was a decrease of the main root length and an increase in the number and length of lateral roots (Figure 4.1). However, an exception to

Figure 4.1 Effect of the inoculation of four bacilli-PGPR strains on root architecture of Chinese cabbage 5 days after inoculation in growth pouches. A-D, main root and lateral root length. E-H, percentage of lateral roots according to four different ranks of length



2-day old seedlings were inoculated with 100 µL of spore suspension. Data correspond to the average of 25 seedlings. Error bars express SE.

Figure 4.2 Seven-day old Chinese cabbage seedlings inoculated with *B. amyloliquefaciens* FZB42 (100 µl of suspension with 10^9 spores/ml). Lateral root length was significantly promoted and more root hairs were observed in inoculated plants



this dual effect was seen with FZB42 at 10^9 spores/ml, which did not result in a significant reduction in main root length, but stimulated production of lateral roots (Figure 4.2). In addition, increases in the number of root hairs were evident in plants inoculated with all the strains at the highest concentration.

3.3. Effect of *B. amyloliquefaciens* FZB42 at different soil P levels

Bacillus amyloliquefaciens FZB42 was selected for this experiment due to its effect in the root pouch assay and because the genome sequence is available, making it a model bacilli-PGPR strain (Chen et al., 2007b). IAA production by this strain was previously reported (Idris et

al., 2007) and, in the present study, production of IAA-like compounds by FZB42 were found to be highly influenced by nutritional factors (Table 4.2).

Table 4.2 Production of IAA-like compounds by *B. amyloliquefaciens* FZB42 on different laboratory media

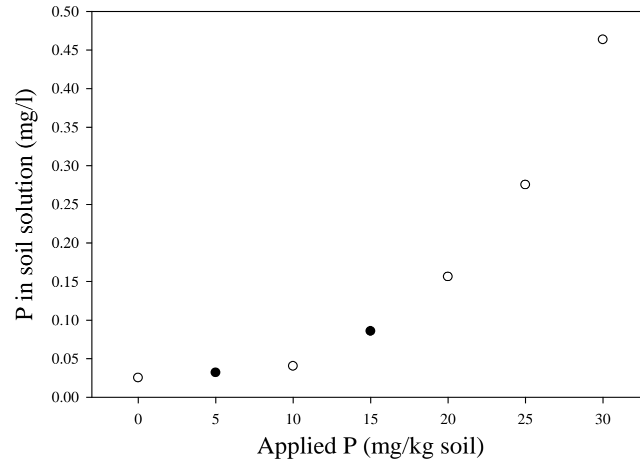
Growth media	IAA equivalents (µg/ml/OD_{600})		
	24h	**48h**	**72h**
LB	0.13 ± 0.03	0.64 ± 0.07	0.90 ± 0.03
LB + Tryptophan	1.04 ± 0.15	1.26 ± 0.14	1.56 ± 0.04
TSB (10%)	0.12 ± 0.07	0.53 ± 0.05	0.32 ± 0.06
TSB (10%) + Tryptophan	1.43 ± 0.12	3.60 ± 0.03	2.54 ± 0.06
TSB (100%)	0.27 ± 0.05	0.89 ± 0.03	1.27 ± 0.09
TSB (100%) + Tryptophan	0.90 ± 0.11	1.67 ± 0.24	1.81 ± 0.12

Data are the average ± standard error from three replications.

The soil P isotherm indicated that the soil mix used for the experiments had 0.025 mg P/l in soil solution. Application of 5 and 15 mg P/kg soil increased the concentration of P in soil solution up to 0.032 and 0.086 mg P/l, respectively (Figure 4.3). Previous experiments showed a highly significant response of Chinese cabbage to these levels of P application to soil (data not shown).

Root architecture of Chinese cabbage was significantly altered by both soil P and bacterial inoculation, but the effect of bacterial inoculation was always dependent on the level and distribution of P in soil (Table 4.3). Soil P was highly significant for all the response variables at both 7 and 14 DAI, while the interaction of soil P and bacterial inoculation was significant for root surface area and number of root tips 7 DAI.

Figure 4.3 Concentration of P in soil solution after application of different amounts of inorganic P to the mixture 1:1 (w/w) soil:sand used in the experiments



Each point represents one single determination. Critical level of P in soil solution to reach 80-95% of maximum yield of cabbage (*Brassica oleracea*) is 0.04 mg P/l (Hue et al., 2000). Filled circles indicate the soil P levels chosen for soil-plant experiments. Response of Chinese cabbage growth at those soil P levels was previously tested in a separate experiment (data not shown).

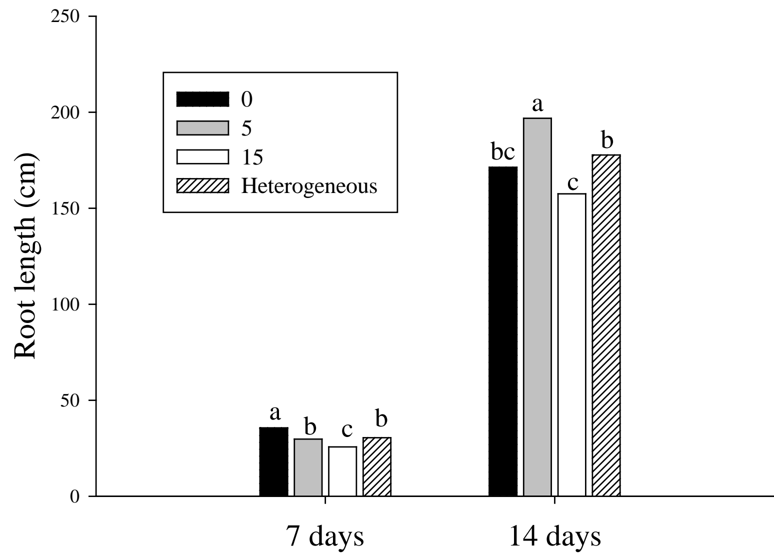
Table 4.3 *P*-values for the analysis of variance of the effects of soil P level and inoculation of *B. amyloliquefaciens* FZB42 on root architecture of Chinese cabbage 7 and 14 days after bacterial inoculation

Effect	7 days after inoculation			14 days after inoculation	
	Total root length	Root surface area	Number of root tips	Total root length	Root surface area
Soil P	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Bacterial inoculation	0.325	0.097	0.524	0.179	0.094
Soil P x Bacterial inoculation	0.452	0.001*	0.032*	0.225	0.127

Soil P included four different levels: addition of 0, 5, and 15 mg P/kg soil with homogeneous distribution, and stratified soil P concentration. Bacterial inoculation was performed with 100 µl of spore suspension at 5 different concentrations (10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml) and a non-inoculated control. Data are from two separate experiments, each having 5 replications per treatment combination. *P*-values followed by an asterisk are considered to be statistically significant.

Total root length was only significantly affected by soil P (Figure 4.4). At 7 DAI, the largest root system occurred at the lowest level of P, while the shortest root system occurred at the highest level of P. At 14 DAI, total root lengths at the two soil P levels were similar, but the largest root systems occurred at 5 mg P/kg soil (Figure 4.4).

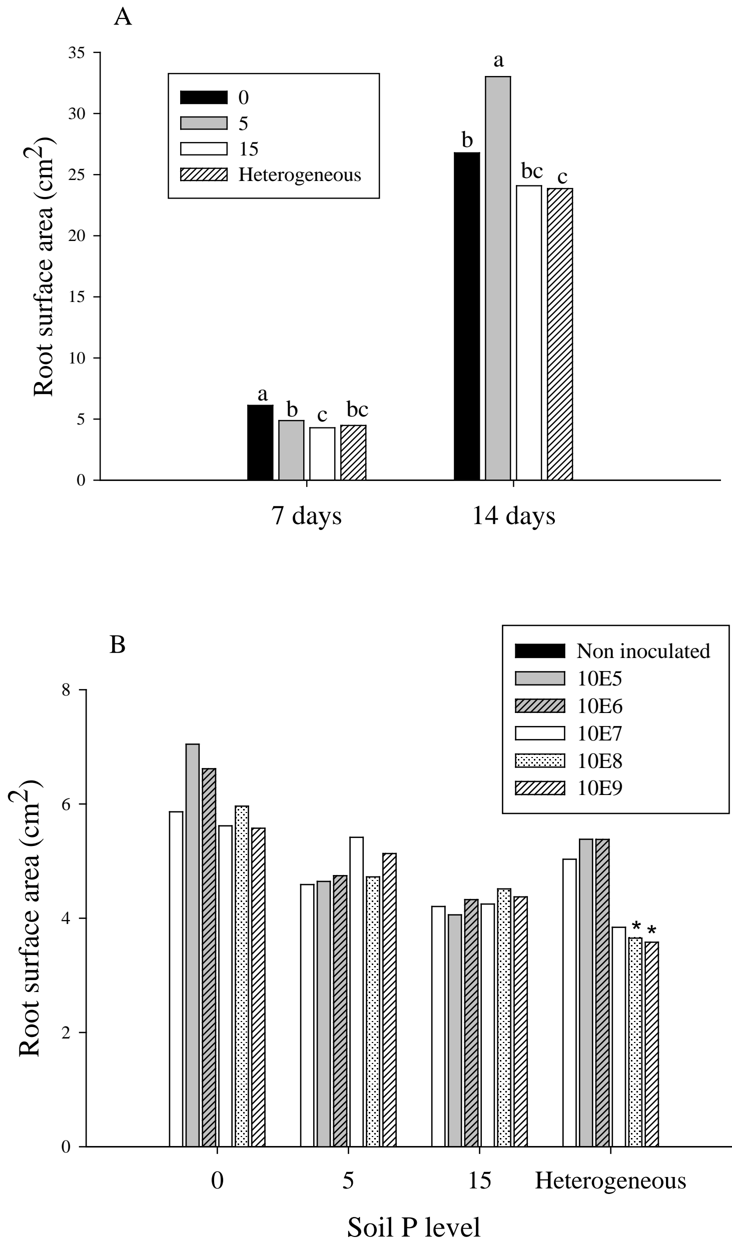
Figure 4.4 Response of Chinese cabbage root length to three homogeneous levels and one heterogeneous level of soil P, 7 and 14 days after planting



Soil P levels corresponded to the addition of 0, 5, and 15 mg P/kg soil distributed homogeneously, and one level with heterogeneous distribution of P (higher concentration in topsoil). Averages are expressed as the least squares means. Those with the same letter within the same evaluation time are not significantly different (P-value > 0.05) according to simulate adjustment of GLIMMIX procedure. Data are from two separate experiments, each having five replicates per treatment combination. ANOVA had 212 and 214 degrees of freedom for the evaluation at 7 and 14 days, respectively. No significant effect or interaction with bacterial inoculation was found (see Table 4.3).

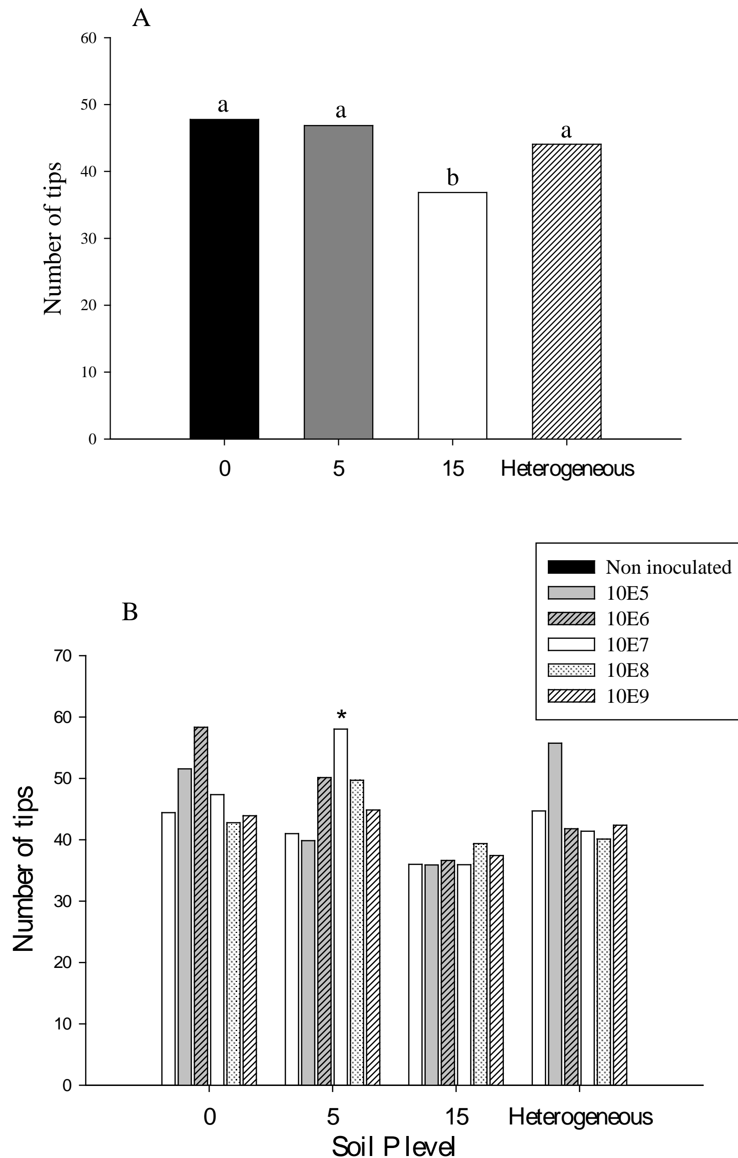
With root surface area, soil P had a significant effect at both evaluation times, while the interaction of soil P and bacterial inoculation was significant at 7 DAI (Table 4.3). Root surface area was stimulated under P-deficient conditions at 7 DAI, but, at 14 DAI, addition of 5 mg P/kg soil produced the highest root surface area (Figure 4.5A). When compared to the non-inoculated control, bacterial inoculation had a significant effect only in the treatment with heterogeneous distribution of soil P 7 DAI. Inoculation with FZB42 at 10^8 and 10^9 spores/ml significantly reduced root surface (Figure 4.5B).

Figure 4.5 Effect of soil P level and inoculation with *B. amyloliquefaciens* FZB42 on root surface of Chinese cabbage. A, main effects for soil P levels at 7 and 14 days after inoculation. B, bacterial effect at each soil P level 7 days after inoculation



Soil P levels corresponded to the addition of 0, 5, and 15 mg P/kg soil distributed homogeneously, and one level with heterogeneous distribution of P (highest concentration in topsoil). Bacterial inoculation was performed with 100 µl of spore suspension at 5 different concentrations (10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml) and a non-inoculated control. Averages are expressed as the least squares means. In A, those values with the same letter within the same evaluation time are not significantly different (*P*-value > 0.05) according to simulate adjustment of GLIMMIX procedure. In B, values with asterisk are significantly different from the non-inoculated control at the same P level (Dunnett's test $P < 0.05$). *P*-values for the *F*-tests are shown in Table 4.3. Data are from two separate experiments, each having five replicates per treatment combination. ANOVA for the evaluation 7 and 14 days after treatment had 212 and 215 degrees of freedom, respectively.

Figure 4.6 Effect of soil P level and inoculation with *B. amyloliquefaciens* FZB42 on the number of Chinese cabbage root tips 7 days after inoculation. A, main effects for soil P levels. B, bacterial effect at each soil P level



Soil P levels corresponded to the addition of 0, 5, and 15 mg P/kg soil distributed homogeneously, and one level with heterogeneous distribution of P (highest concentration in topsoil). Bacterial inoculation was performed with 100 µl of spore suspension at 5 different concentrations (10^5, 10^6, 10^7, $10^{8,}$ and 10^9 spores/ml) and a non-inoculated control. Averages are expressed as the least squares means. In A, those values with the same letter are not significantly different (P-value > 0.05) according to simulate adjustment of GLIMMIX procedure. In B, values with asterisk are significantly different from the non-inoculated control at the same P level (Dunnett's test $P < 0.05$). P-values for the F-tests are shown in Table 4.3. Data are from two separate experiments, each having five replicates per treatment combination. ANOVA had 211 degrees of freedom.

For root tips, which was only determined at 7 DAI, soil P level and the interaction of soil P and bacterial inoculation was significant. The lowest number of root tips corresponded to the highest soil P level (Figure 4.6A). In addition, inoculation with FZB42 promoted the number of root tips only with addition of 5 mg P/kg soil and at 10^7 spores/ml (Figure 4.6B).

Changes in shoots of Chinese cabbage were also detected at 14 DAI. Only soil P level had a significant effect on fresh shoot weight, and shoot P_i content was significantly affected by the interaction between soil P and bacterial inoculation (Table 4.4). Bacterial inoculation caused a significant reduction of P_i in the shoot of plants grown with addition of 5 mg P/kg soil, and this was dependent on the inoculum concentration used, occurring at 10^6, 10^7, and 10^9 spores/ml. Conversely, FZB42 inoculation at 10^9 spores/ml significantly increased shoot P_i content when P was heterogeneously distributed in the soil and P concentration was higher in the top soil (Figure 4.7). In contrast, bacterial inoculation did not have a significant effect when P was not added or when P was sufficient to support plant growth (addition of 15 mg P/kg soil). Bacterial inoculation did not significantly increase shoot fresh weight in spite of the increase in shoot P_i content. However, correlation analysis showed that bacterial inoculation and shoot fresh weight were positively correlated ($r = 0.806$, $P < 0.001$; Figure 4.8). In addition, relative decreases in fresh shoot weight occurred at 5 mg P/kg soil, while increases occurred with heterogeneous distribution of P (Figure 4.7).

Table 4.4 ***P*-values for the analysis of variance of the effects of soil P level and inoculation with *B. amyloliquefaciens* FZB42 on fresh shoot weight (FSW) and shoot P_i content of Chinese cabbage 14 days after bacterial inoculation**

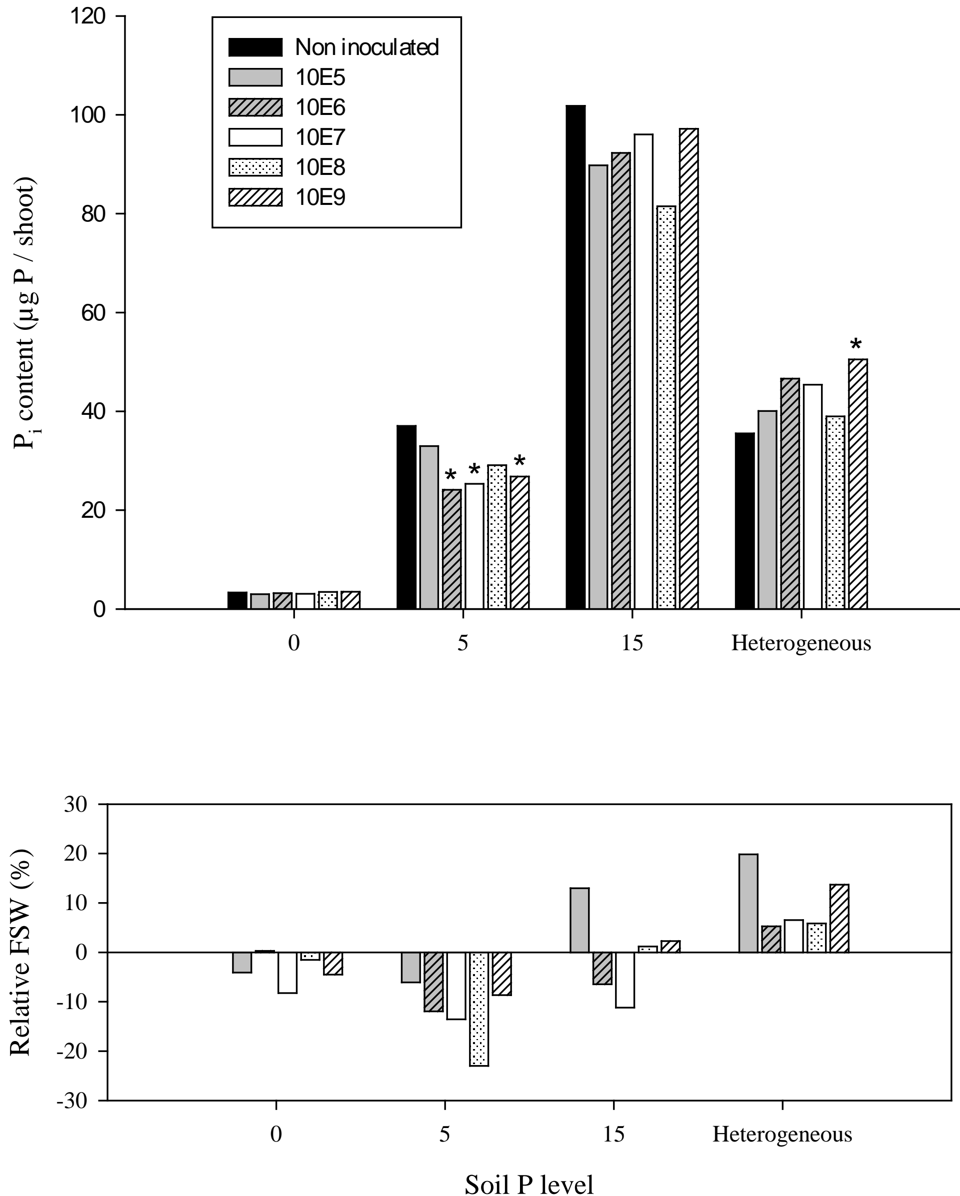
Effect	Fresh shoot weight	Shoot P_i content
Soil P	< 0.001*	< 0.001*
Bacterial inoculation	0.105	0.556
Soil P x Bacterial inoculation	0.337	0.007*

Soil P included four different levels: addition of 0, 5, and 15 mg P/kg soil with homogeneous distribution, and stratified soil P concentration. Bacterial inoculation was performed with 100 µL of spore suspension at 5 different concentrations (10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml) and a non-inoculated control. Data are from two separate experiments, each having 5 replications per treatment combination. *P*-values followed by an asterisk are considered to be statistically significant.

4. Discussion

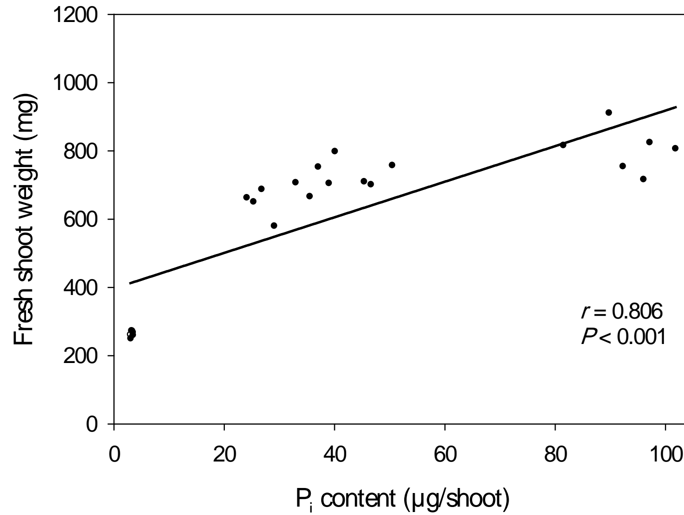
In this study, we show that bacilli PGPR induce changes in root architecture of Chinese cabbage seedlings in growth pouches (Figures 4.1 and 4.2). In addition, we demonstrate that inoculation of the model bacilli-PGPR strain FZB42 also modifies root architecture (Figures 4.5 and 4.6) and P uptake (Figure 4.7) in a soil-plant experimental system, but this effect is modulated by the content and distribution of P in soil. Root architecture is recognized as a key factor for plant nutrition and water uptake (Pierret et al., 2007). The importance of root architecture in plant productivity relies on the fact that many soil resources are heterogeneously distributed, so that spatial deployment of the root system strongly determines the capacity of the plant to exploits those resources (de Dorlodot et al., 2007; Lynch, 1995). As stated by Hutchings and John (2004), most of the studies on plant growth have been based on experiments conducted under spatially homogeneous conditions which does not give a proper understanding of plant responses in natural environments. Nutrients in soil are heterogeneously distributed, so that pronounced gradients occur with soil depth (Jobbágy and Jackson, 2001), even over a scale of

Figure 4.7 Effect of the inoculation with *B. amyloliquefaciens* FZB42 on shoot P_i content and fresh shoot weight of Chinese cabbage grown at 3 levels of P homogeneously distributed in soil and 1 with heterogeneous distribution (highest concentration in topsoil)



Determination 14 days after bacterial inoculation. Soil P levels corresponded to the addition of 0, 5, and 15 mg P/kg soil distributed homogeneously, and one level with heterogeneous distribution of P (higher concentration in top soil). Bacterial inoculation was performed with 100 µL of spore suspension at 5 different concentrations (10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml) and a non-inoculated control. Averages of shoot P_i content are expressed as the least squares means. Those values with asterisk are significantly different from the non-inoculated control at the same P level (Dunnett's test $P < 0.05$). Relative FSW is expressed as the percentage of increase or decrease compared to the non-inoculated control. *P*-values for the *F*-tests are shown in Table 4.4. Data are from two separate experiments, each having five replicates per treatment combination. ANOVA for the FSW and shoot P_i content had 214 and 215 degrees of freedom, respectively.

Figure 4.8 Pearson product-moment correlation coefficient for the values of fresh shoot weight and shoot P_i content of 14-day old Chinese cabbage plants from the soil-plant experiment



Each point represents the least square means for 10 individual determinations (n = 24). Non-inoculated plants and plants inoculated with different concentrations of *B. amyloliquefaciens* FZB42 under 4 different soil P levels are included.

centimeters (Lynch, 1995). This is especially true for P which has a very low mobility in soil (Bieleski, 1973) and the concentration of which is greater in surface horizons due to sorption of added P, greater biological activity, and accumulation of organic material in surface layers (Sharpley, 1995). Heterogeneous distribution of P is even more dramatic in highly weathered tropical soils such as oxisols and ultisols where P availability is highly limited and concentrated in a thin surface layer (Lynch, 1995).

The potential of PGPR for biofertilization is widely reported (Banerjee et al., 2006). Beneficial effects reported for PGPR include increases in a number of parameters such as germination rates, root and shoot growth (length and/or weight), yield, leaf area, content of chlorophyll, nutrients, and protein, as well as inducing delayed leaf senescence and tolerance to drought and other stress factors (Lucy et al., 2004; Yang et al., 2009). These benefits have been

observed in all bacterial groups including bacilli (Adesemoye et al., 2009; Krebs et al., 1998; Yao et al., 2006), which are particularly important from a practical point of view. This importance stems from their easy mass production and unique formation of highly resistant endospores, characteristics that make them rather feasible to be formulated and integrated in commercial agriculture (Mathre et al., 1999; Ongena and Jacques, 2008).

The mechanisms by which PGPR enhance nutrient status of host plants have been classically divided into five groups: 1) biological N_2 fixation, 2) increasing the availability of nutrients in the rhizosphere, 3) inducing increases in root surface area, 4) enhancing other beneficial symbiosis of the host, and 5) combination of modes of action (Vessey, 2003). Specifically regarding P nutrition, almost all the attention has been given to P solubilization from organic and inorganic sources (Richardson, 2007). A great number of studies report the isolation of bacterial strains capable of solubilizing P *in vitro* and the increase of plant growth when they are used as inoculants (Rodriguez and Fraga, 1999). However, a causal link between the capacity to solubilize P and to promote plant growth is not clear. First, because several potentially beneficial traits other than P solubilization, *e.g.* IAA production, are commonly present in the same strain making it difficult to estimate the contribution of each individual mechanism (Banerjee et al., 2006). Second, contradictory results are often obtained, so that no increases in plant growth and/or P uptake are sometimes seen when bacterial P solubilizers are applied (de Freitas et al., 1997). Nonetheless, P solubilization by PGPR keeps attracting much attention because of its potential. In the group of plant-associated bacilli, this trait seems to be common since P-solubilizing strains are frequently isolated (Nautiyal, 1999; Vazquez et al., 2000) and, in our results, the frequency of *in vitro* P solubilizers was high (85%).

Our results suggest that, under uneven distribution of P in soil, PGPR inoculation could alter root architecture during early growth and allow the plant to explore topsoil more efficiently. This is particularly important for P given its low mobility in soil and, under natural conditions, its higher concentration near the soil surface (Bieleski, 1973; Jobbágy and Jackson, 2001). However, side effects could be originated from those changes as shallower roots systems tend to be more susceptible to drought (Walk et al., 2006). All these effects must still be tested in well-characterized fields where P content in soil profile is properly determined and for longer periods of plant growth, since the present study was conducted in artificial conditions and only evaluated the effects during early growth. Classically, when PGPR-induced changes of root system have been related to increases in plant nutrient uptake, the total root surface area is the main variable considered (Banerjee et al., 2006; German et al., 2000; Vessey, 2003). However, in those experiments, substrates with a homogeneous nutrient distribution were used, which, as pointed by Hutchings and John (2004), does not reflect the heterogeneous conditions existing in natural soils.

Results obtained here suggest that modification of the root system favoring lateral root development could increase the plant's scavenging capacity for soil P when this is heterogeneously distributed (Figure 4.7). Results of the growth pouch assays showed that inoculation of bacilli-PGPR strains stimulated lateral root growth of Chinese cabbage in a concentration-dependent manner. In studies conducted with beans, significant correlation between branching angle of roots in growth pouches and seed yield in low P soils were found (Bonser et al., 1996). Subsequently, several other investigations demonstrated that bean

genotypes with more lateral root development, and thus a shallower root system, are more efficient in foraging topsoil for P (Lynch and Brown, 2001; Rubio et al., 2003).

In our experiments, we found thatFZB42 inoculation also modifies root architecture in soil, but this is greatly modulated by soil P (Table 4.3; Figures 4.5 and 4.6). The significant reduction of root surface area upon bacterial inoculation of plants grown in a soil with a gradient of P, while root length remained the same, indicates that thinner roots were produced. This is consistent with the finding that lateral roots observed in Chinese cabbage in both root pouch and soil experiments were thinner. In addition, the significantly higher P uptake by plants in these tests indicates that root activity was improved in the portion of soil where more P was present, *i.e.* the topsoil, compared to the non-inoculated plants. Such significantly higher P scavenging of topsoil in inoculated plants also opens possibilities for increasing the efficiency of surface-applied fertilizers, especially those that are poorly mobile like P.

Most of the studies on root stimulation during early growth by PGPR have focused on elongation of the main root and do not take into account the heterogeneous nutrient distribution occurring in soil (Li et al., 2000; Patten and Glick, 2002). One of the few publications reporting increases in lateral root development describes the effect of the *Phyllobacterium* sp. strain 29-15 on early stages of *Brassica napus* (Larcher et al., 2003). In this study, inoculation of 29-15 in a soilless system promoted lateral root density through the primary root and growth rate of lateral in a concentration-dependent manner, like in our work. Subsequently, in other study also conducted *in vitro*, it was found that nitrate-dependent control of root architecture and N

nutrition of *Arabidopsis* are altered by inoculation with a closely related strain, *Phyllobacterium* STM196 (Mantelin et al., 2006). Those two studies were conducted in soilless systems

The effect of PGPR on root architecture is usually attributed to production of phytohormones or the regulation of their contents in plant tissue (Glick et al., 2007). In gram-negative PGPR, root stimulation has been widely linked to production of IAA (Dobbelaere et al., 1999; Patten and Glick, 2002) and cytokinins (García de Salamone et al., 2006) as well as reduction in the levels of ethylene by the action of ACC-deaminase (Li et al., 2000). For bacilli PGPR, in contrast, evidence is less available. For instance, IAA produced by FZB42 was involved in growth promotion of the aquatic plant *Lemna minor* (Idris et al., 2007). However, no root measurements were done in that study. Regarding cytokinins, *Arabidopsis* root system responded to cytokinins produced by *B. megaterium* UMCV1 in experiments conducted in agar and without direct inoculation (Ortíz-Castro et al., 2008). In the case of ACC-deaminase, production was detected in three isolates of *B. circulans*, *B. firmus*, and *B. globisporus,* and inoculation of those strains promoted plant growth and root elongation (Ghosh et al., 2003).

From the results obtained in our screening, production of ACC-deaminase seems to be uncommon in plant-associated bacilli since none of the tested strains produced the enzyme (Table 4.1). In contrast, IAA-like compounds were produced by 68% of the strains that showed various levels of production. The concentration-dependent effect seen with FZB42 could be explained by the action of IAA, a phenomenon that has been reported in other PGPR (Dobbelaere et al., 1999). However, the absence of primary root inhibition at the highest concentration, while lateral roots were still stimulated, suggests that other compounds may be

acting simultaneously. In addition, we observed that production of IAA in FZB42 is highly dependent on growth medium (Table 4.2). This could suggest that soil nutrient content impacts IAA production by FZB42 in the rhizosphere and is related to the great influence that soil P displayed on bacterial inoculation effects in Chinese cabbage. This suggestion is not easy to test because it will require separating the effect of soil P on the bacterium from that on the plant, which could be rather problematic.

Interestingly, changes in root architecture observed when bacilli-PGPR were inoculated on Chinese cabbage resemble those reported for *Arabidopsis* (Williamson et al., 2001) and beans under conditions of P limitation (Liao et al., 2001). A common factor in the root architecture changes induced by both PGPR inoculation and P deficiency is the role played by IAA, for which a great deal of evidence supports that it is responsible for lateral root formation (Casimiro et al., 2001; Gaspar et al., 2002; Woodward and Bartel, 2005). In *Arabidopsis*, stimulation of lateral roots and inhibition of primary root produced by P deficiency are mediated by IAA (Lopez-Bucio et al., 2002; Perez-Torres et al., 2008). In beans, the number of basal roots, which increases under P-limiting conditions (Bonser et al., 1996), is also increased by addition of IAA (Remans et al., 2008). Therefore, bacterial inoculation and soil P level produce not only similar effects, but they also seem to share common mechanisms. This creates a highly complex interaction that additionally depends on the plant's responsiveness to IAA and that will determine the inoculation outcome.

Modulation of PGPR performance by soil P content was described in common bean. Root architecture was not evaluated in those studies but significant increases in number of nodules and

plant weight were obtained when *Azospirillum brasilense* Sp245 and *B. subtilis* LMG7135, tested at a single concentration, were inoculated under high P conditions (Remans et al., 2007). In contrast, under low P fertilization, *A. brasilense* Sp245 caused significant reductions of those same variables, and *B. subtilis* LMG7135 did not have any effect. The opposite effect was seen with *Pseudomonas putida* UW4, which promoted plant weight and the number of nodules under low P content, but not when P level was high (Remans et al., 2007). This strain-dependent effect was correlated to the fact that Sp245 and LMG7135 are IAA producers whereas UW4 produces ACC deaminase. Consistently, the effect of PGPR inoculation and exogenous application of IAA depended on root responsiveness to auxins, a phenomenon that was also modulated by the level of P in the substrate (Remans et al., 2008).

Unexpectedly, FZB42 inoculation at concentrations of 10^6, 10^7, and 10^9 cfu/ml had a negative effect when 5 mg P/kg soil were applied, *i.e.*, 0.032 mg P/l in soil solution (Figures 4.3 and 4.7). Significant reductions in Pi content were observed at those inoculum concentrations and they were associated with lower fresh shoot weight. Data suggest that this effect was not related to changes in root architecture as no bacterial effect was found for any of the two root variables that were measured (Table 4.3). However, a root-mediated effect cannot be ruled out because other variables such as root hair formation could have been affected.

One possible explanation for the reduction in plant P_i content upon bacterial inoculation is the immobilization of rhizosphere P by actively growing FZB42 cells. Under this soil P content, root length was maximized in comparison to the rest of soil P treatments (Figure 4.4). This indicates that carbon allocation to roots was the highest at this soil P level, and thus exudation should have

also been high (Lu et al., 1999). High carbon availability stimulates bacterial growth and increases bacterial demand for P (Vrede et al., 2002). Since soil P content was still suboptimal, available P could have been insufficient to support both fast growing inoculated bacteria and plant growth. This would have led both bacteria and plant to compete for this limiting resource.

Another possible explanation is that, under what are still suboptimal conditions for plant growth, the demand for carbon sources by inoculated bacteria could have resulted in a higher cost for the plant. In well-recognized beneficial symbionts like mycorrhizal fungi, evidence suggests that the symbiotic relationship with the plant can go from mutualistic to parasitic according to the environmental conditions (Johnson et al., 1997). Here, the result of plant-microorganism interaction is understood as the balance between the cost and the benefits of such interaction. When the net cost of the symbiosis exceeds net benefits, this interaction is parasitic. This is, clearly, a complex phenomenon that deserves further investigation that should be based on ecophysiological concepts.

Our findings contribute to characterize some basic factors that could be behind the variability in PGPR results. One major limitation for widespread use of PGPR in agriculture is the inconsistent results that are frequently obtained in the field. A number of factors such as soil moisture, native soil microbiota, colonization, soil pH and nutrient content, plant host, among others, are commonly enumerated to explain this variability. However, the contribution of each of those factors is poorly characterized, hampering the prediction or optimization of PGPR performance. Given the practical potential of easily formulated-bacilli PGPR, such characterization is particularly important for this PGPR group. In this study, results suggest that

soil P distribution could be related to PGPR performance. In addition, soil P content strongly modulated the bacterial effect on P uptake by Chinese cabbage during early growth, leading from promotion to depression. Duration of those effects is still to be evaluated, but new aspects are brought to discussion.

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