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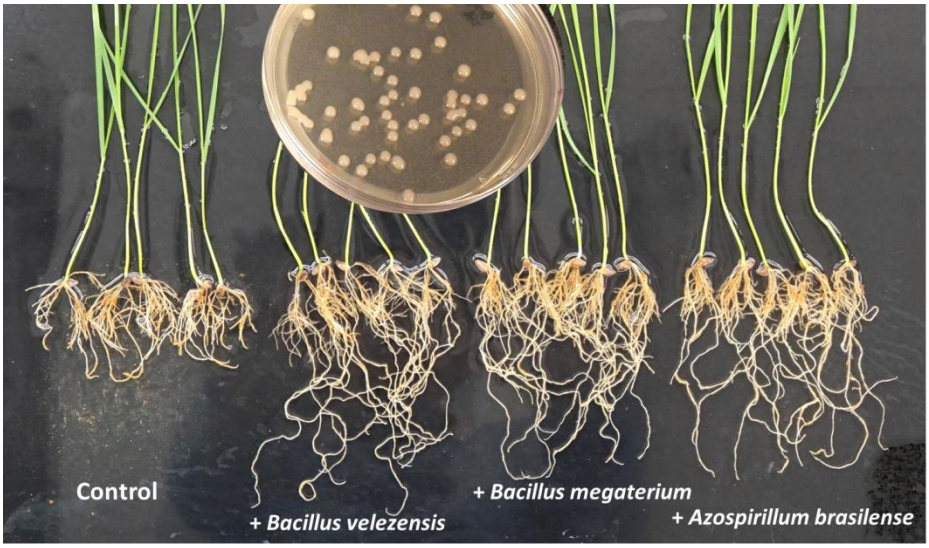
Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake under contrasted N supplies

Nguyen Minh Luan



Promoteurs: Pierre Delaplace - Patrick du Jardin

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Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake under contrasted N supplies

Nguyen Minh Luan

Dissertation originale présentée en vue de l'obtention du grade de docteur en Sciences agronomiques et ingénierie biologique

Promoteurs: Pierre Delaplace - Patrick du Jardin
Année civile: 2018

I dedicate this work to my parents
for their love, their support
and for all the things they sacrificed for helping me

Summary

Minh Luan Nguyen (2018) **Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake under contrasted nitrogen supplies** (PhD Thesis). Gembloux, Belgique, University of Liège – Gembloux Agro-Bio Tech, 170 p., 9 tabl., 22 fig.

Plant growth-promoting rhizobacteria (PGPR) are increasingly used as microbial biostimulants. Hereby, the capacities of PGPR to promote plant growth and nutrient uptake in wheat were evaluated with contrasted mineral N fertilization rates under gnotobiotic, greenhouse, and field conditions. Six PGPR strains were employed for the tests, including three laboratory strains *Bacillus velezensis* GB03 (BveGB03), *B. megaterium* SNji (BmeSNji), *Azospirillum brasilense* 65B (Abr65B), and three commercially formulated trains, *B. velezensis* IT45, FZB24, and FZB42.

Under gnotobiotic conditions using sterile soil, all strains significantly increased plant biomass 14 days after inoculation irrespective of the N fertilization rates. Under greenhouse conditions, the highest growth promotion was recorded under moderate N supply (50N), followed by full N dose (100N), while no significant effect of the inoculant was observed in the absence of N fertilizer (0N). At 50N, the biomass was most significantly increased in specific plant parts, i.e. in roots (increase up to +45%) 30 days after inoculation with Abr65B and in the ears (19–23% increase) with BveGB03, BmeSNji, Abr65B 60 days after inoculation. At 0N, FZB24 was able to significantly increase root biomass of spring wheat up to +31% 30 days after incoculation. Under field conditions, FZB24 significantly increased grain yields by 983 kg ha^{-1} (14.9%) as compared to non-inoculated controls at 0N in 2014 field trials. However in 2015 field trials, FZB24 was not able to replicate the previous positive results, likely due to the low temperatures occurring during and after the inoculations at tillering stage.

The increase in plant biomass caused by PGPR inoculation was paralleled with lowered concentrations of several nutrients in the same organs of plants growing under greenhouse conditions. Specifically, the increases in root and ear biomass caused by BmeSNji, Abr65B were paralleled with lowered concentrations in N, P, Mn, and Cu (organ- and strain-specific). Regarding IT45 and FZB24 inoculations, when the increase in biomass was lower, only two nutrients (P and K) exhibited a lowered concentration while other nutrient (Fe, Mn, Zn, and Cu) concentrations were significant increased. In contrast, the highest increases in plant biomass stimulated by PGPR inoculations goes along with higher total nutrient content and nutrient uptake efficiency.

The results are discussed in the perspective of PGPR implementation in contrasted cultivated systems and their interaction with fertilizer application.

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Résumé

Minh Luan Nguyen (2018) **Effets biostimulants de rhizobactéries sur la croissance et le prélèvement de nutriments du blé dans des contextes d'apports azotés contrastés** (Thèse de doctorat). Gembloux, Belgique, Université de Liège – Gembloux Agro-Bio Tech, 170 p., 9 tabl., 22 fig.

Les rhizobactéries promotrices de la croissance végétale (PGPR : Plant Growth Promoting Rhizobacteria) sont de plus en plus utilisées comme biostimulants microbiens. Dans le présent travail, les capacités de PGPR à améliorer la croissance et le prélèvement de nutriments du blé ont été évaluées dans des contextes d'apports en fertilisants azotés contrastés en conditions gnotobiotiques, sous serre ou en plein champ. Six souches PGPR ont été utilisées pour les essais, à savoir 3 souches cultivées en laboratoire [*Bacillus velezensis* GB03 (BveGB03), *B. megaterium* SNji (BmeSNji), *Azospirillum brasilense* 65B (Abr65B)], et trois ouches disponibles commercialement (*B. velezensis* IT45, FZB24, et FZB42).

En conditions gnotobiotiques utilisant un sol stérilisé, toutes les couches accroissent la biomasse des plantules significativement 14 jours après l'inoculation, quel que soit le niveau d'apport azoté. Sous serre, la plus forte promotion de croissance a été enregistrée avec des apports azotés modérés (50N) et, dans une moindre mesure, élevés (100N), alors qu'aucun effet significatif de l'inoculant microbien n'était observé en l'absence de fertilisation azotée (0N). Sous apport modéré (50N), l'augmentation de biomasse est observée dans certains organes de la plante, à savoir les racines (augmentation jusqu'à 45%) 30 jours après inoculation avec Abr65B et dans les épis (augmentation de 19 à 23%) avec BveGB03, BmeSNji et Abr65B 60 jours après inoculation. Sans apport azoté (0N), FZB24 augmente significativement la biomasse racinaire du blé de printemps de 31%, 30 jours après inoculation. Dans des conditions de plein champ, FZB24 augmenta significativement le rendement en grain de 983 Kg ha-1 (+ 14,9% par rapport au contrôle non fertilisé) lors des essais de 2014. Ces résultats n'ont cependant pas pu être reproduits en 2015, probablement à cause de températures trop basses après l'inoculation au stade tallage.

L'augmentation de la biomasse des organes précités suite à l'inoculation des PGPR s'accompagne d'une diminution de concentration de plusieurs nutriments sous serre. Plus spécifiquement, l'augmentation des biomasses des racines et des épis induite par BmeSNji et Abr65B vont de pair avec des concentrations réduites en N, P, Mn et Cu dont l'ampleur est variable selon la souche et l'organe considéré. En ce qui concerne les inoculations avec IT45 et FZB24, lorsque l'augmentation de

biomasse est plus faible, seulement deux éléments minéraux (P et K) présentent une concentration réduite alors que les concentrations d'autres éléments (Fe, Mn, Zn et Cu) augmentent significativement. Contrairement aux mesures de concentrations, les plus fortes augmentations de biomasse induites par l'inoculation de PGPR s'accompagnent d'un contenu total en éléments minéraux accru et d'une plus forte efficience de prélèvement des nutriments.

Ces résultats sont discutés dans la perspective d'une intégration de l'inoculation de PGPR dans des systèmes de culture contrastés en considérant leur interaction avec l'application de fertilisants.

Acknowledgments

Many people have contributed to the achievement of this PhD thesis in many ways. All these actors must be acknowledged hereafter:

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works and the fantastic times together. It was so fortunate to have you by my side in our lab.

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Nguyen Minh Luan

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List of Abbreviation

Abr65B: *Azospirillum brasilense* 65B

ACC: 1-aminocyclopropane-1-carboxylate

BveFZB24: *Bacillus velezensis* FZB24

BmeSNji: *Bacillus megaterium* SNji

BveFZB42: *Bacillus velezensis* FZB42

BveGB03: *Bacillus velezensis* GB03

BveIT45: *Bacillus velezensis* IT45

CFU: Colony-forming unit

CKs: Cytokinins

ET: Ethylene

GAs: Gibberellins

IAA: Indole-3-acetic acid

IAM: Indole 3-acetamide

IBA: Indole-3-butyric acid

ILA: Indole-3-lactic acid

IPDC: Indole-3-pyruvate decarboxylase

IPyA: Indole-3-pyruvate

ISR: Induced systemic resistance

JA: Jasmonic acid

MAMPs: Microbe-associated molecular patterns

NFP: Nod factor perception

NO: Nitric oxide

NUE: Nutrient uptake efficiency

PAA: Phenylacetic acid

PGPR: Plant growth-promoting rhizobacteria

PLS: POLARIS

TAM: Tryptamine

VOCs: Volatile organic compounds

General introduction

This PhD thesis was conducted at the Plant Biology Laboratory, which is a part of Gembloux Agro-Bio Tech, the Faculty of agricultural sciences and bioengineering of the University of Liège. This project is funded by the AgricultureIsLife research platform and under the supervison of Dr. Pierre Delaplace and Dr. Prof. Patrick du Jardin.

AgricultureIsLife research platform was founded in Gembloux Agro-Bio Tech in order to provide practical solutions and develop the tools, techniques and knowledges of sustainability according to the ongoing changes in the agricultural systems currently. For this purpose, AgricultureIsLife encompasses four research axes: Axis 1 – What is the performance of non-conventional agro-ecosystems? Axis 2 – How can we best valorize agricultural residues? Axis 3 – Which are the new tools and technology to develop for agriculture? Axis 4 – What can be an alternative destiny or valorisation of agricultural products?

This PhD thesis belongs to AgricultureIsLife research axis 3. It was lanched to develop and implement the new tools to reduce the use of chemical fertilizers as well as their agro-ecological threats in the prospect of sustainable agriculture. Nowadays, maintaining the soil fertility is a major concern in the intensive production of crops. A better understanding of root ecosystem, the root physiology and their biotic interactions within the rhizosphere could be the solution to maintain soil fertility while crop biomass is massively exported from the farms. Plant growth and health are deeply influenced by microbial communities in soil. Noticeably, some microorganisms are known to (i) promote root growth and modify the root system architecture, (ii) solubilize mineral nutrients, (iii) fix atmosphere nitrogen, and (iv) enhance the nutrient uptake from the soil. Plant growth-promoting rhizobacteria (PGPR) are one of the major biostimulant classes (du Jardin P., 2015) and are able to stimulate root growth, enhance mineral availability, and nutrient use efficiency in crops (Bhattacharyya and Jha, 2012; Nguyen et al, 2018). PGPR-based biostimulants could therefore reduce the demand of chemical fertilizer and lessen their negative environmental impacts.

The aims of this PhD project are (1) to screen PGPR strains to enhance wheat growth and productivity, and (2) to understand the impact of PGPR on the nutrient uptake efficiency and the impact of environmental factors on the plant-PGPR interaction. In this PhD project, several PGPR-based biostimulants have been collected and screened for their plant growth promoting capacity under gnotobiotic,

greenhouse, and field conditions. In parallel, the impacts of N fertilization rates on the performance of PGPR are also assessed in order to optimize the agricultural practices under field conditions.

This PhD thesis is organized as follow:

Chapter 1: a general introduction presents the definition of PGPR-based biostimulants and their updated mode-of-action, and an overiew of their current implementation under field conditions as well as the challenges for the production, formulation application, and regulatory framework;

Chapter 2: the aims of the thesis

Chapter 3: the strategic choices that justify the selected materials and methods

Chapter 4: the evaluation of the efficiency of PGPR on wheat growth and nutrient uptake, and their interaction with N application and plant development

Chapter 5: the evaluation of the biostimulant effects of PGPR strains belonging to *Bacillus velezensis*, on wheat from in vitro towards field conditions and their interaction with fertilizer and temperature;

Chapter 6: General discussion, conclusion, and perspectives;

Chapter 7: a last chapter presents a list of scientific communications.

1

Bibliographical Introduction

1 Bibliographical Introduction

Most parts of this chaper were published in this following article:

Géraldine Le Mire*, Minh Luan Nguyen*, Bérénice Fassotte, Patrick du Jardin, François Verheggen, Pierre Delaplace**, M. Haissam Jijakli**, 2016. **Review: Implementing plant biostimulants and biocontrol strategies in the agroecological management of cultivated ecosystems.** *Biotechnologie Agronomie, Société et Environnement*, 20(S1), 299-313. *These authors contributed equally to this work. **Co-last authors. https://orbi.uliege.be/handle/2268/188662. The new sections "Mode of actions", "Impact of PGPR on nutrient enrichment and nutrient uptake efficiency", and "PGPR implementation under greenhouse and field conditions" were later added beside the sections of the published article.

The decline in natural resources and the environmental damage inflicted by current agricultural practices have become major limitations in conventional agriculture. Against this background, agroecology offers an important scientific approach that takes into account the current societal concerns linked to agriculture, economy and, in particular, the environment. By using ecological principles, it aims at studying and designing agricultural systems based on the interactions of their main biophysical, technical and socioeconomic components (European Commission 2012).

The human population is estimated to reach 9.6 bilions by the year 2050, and this consequently resulted in a requirement of at least double amount of our current agricultural production (Wilson, 2003; Bruinsma, 2009). In order to secure the global food production for a fast-growing population, the cropping systems need to be shifted to a way that maximizes the crop productivity while minimizes the resource inputs, especially for chemical fertilizers and pesticides (Tilman et al., 2002). The development of new green technologies has led to greater research strongly focussed on the use of agroecological principles to minimize potentially harmful chemical inputs and manage ecological relationships and agro-biodiversity. The past decade has seen the emergence of technological tools developed to promote sustainable agroecosystems. The enhancement of plant tolerance to numerous abiotic stresses is increasingly being supported by biostimulant products, as preferred alternatives to chemical fertilizers. Biostimulants include living microorganisms, namely plant growth-promoting fungi (PGPF) and rhizobacteria (PGPR) (P. Bhattacharyya & Jha 2012). PGPR are currently thought to be an effective tool for the biostimulation of plant growth (Bashan et al., 2014; Calvo et al.

2014; Nguyen et al., 2018). Beneficial rhizobacteria associate with the root system and stimulate the growth of host plants, while being fed in turn by root exudates.

This chapter gives an overview of the definition, mode-of-action for plant growth-promoting capacity of PGPR, the impact of PGPR on nutrient use efficiency, and the state-of-the-art of current methods for exploiting and implementing PGPR-based biostimulant products in contemporary agricultural systems. Future applications of PGPR-based biostimulants for sustainable management of cultivated ecosystems are also discussed.

1.1 PGPR-based biostimulants in conventional agriculture

1.1.1 PGPR-based biostimulants

The sustainable management of soil fertility is a major concern given the adverse impact and ecological threats posed by the use of conventional chemical fertilizers (Wezel et al. 2014). In this context, biostimulants represent an interesting alternative. They consist of various substances and microorganisms (humic and fulvic acids, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, protein hydrolysates and amino acids, beneficial fungi and bacteria), which are used to enhance plant growth (du Jardin, 2015). They can increase crop yield by at least 5-10% and improve fertilizer use efficiency by at least 5-25% (European Biostimulants Industry Consortium, 2011). The global market of biostimulants it is projected to increase by 12% annually (Calvo et al. 2014).

Despite their growing use, there is currently no accepted definition of biostimulants, neither by regulatory bodies nor by the scientific community. However, with the revision of current European Union (EU) legislation on fertilizers, there has been some progress. At the same time, a literature review defined a plant biostimulant as « *any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content. By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms*» (du Jardin, 2015) (Figure 1). This definition clearly differentiates biostimulants from biocontrol substances or agents.

In this chapter, we focus on biostimulants in the category of microbial inoculants, particularly PGPR which have been intensively studied in recent decades (Calvo et al. 2014). Plant growth promoting rhizobacteria (PGPR) are difined as soil bacteria that colonize the roots of plants and the rhizosphere, and are therefore called rhizobacteria, following inoculation onto the seeds that are able to enhance plant

growth and biocontrol activities (Kloepper et al., 1880 and 1993). The PGPR-based biostimulants are major components of biofertilizers intended for agricultural use. They have long been commercialized by many manufacturers and applied to various field crops such as maize, rice, soybean, and wheat (Köhl 2010; Pérez-Montaño et al. 2013), as well as to horticultural crops such as tropical, subtropical and temperate fruits and vegetables (Reddy 2014). PGPR-based biostimulants are considered to be easy-to-use agroecological tools for stimulating plant growth and enhancing plant nutrient uptake and abiotic stress tolerance (Walker et al. 2012; Hol et al. 2013; Pérez-Montaño et al. 2013). They can also enhance beneficial symbioses with the host plant.

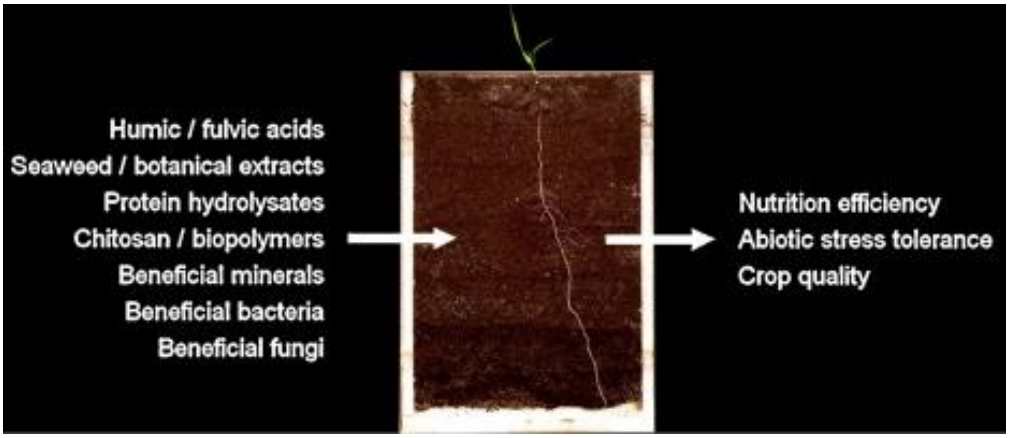

Figure 1. Main categories of plant biostimulants including various substances or microorganisms. They are applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of their nutrients content (du Jardin, 2015). The study of bacterial biostimulants, particularly plant growth-promoting rhizobacteria (PGPR), will be focussed in this thesis

1.1.2 Mode of actions

1.1.2.1 General mode of actions

PGPR are able to stimulate the plant growth and protect plants against abiotic or biotic stresses by several mechanisms (Vacheron et al., 2013, Vejan et al, 2016) (Figure 2). Firstly, the mechanisms relate to the stimulation of plant physiological status and the enhancement of nutrient uptake, such as production of phytohormones

or analogs (i.g. auxins, cytokinins, gibberellins), volatile organic compounds (VOCs, such as 2,3-butanediol, 3-hydroxy-2-butanone, 2-pentylfuran, N,N-dimethyl-hexadecanamine, CO2, 13-tetradecadien-1-ol, 2-butanone and 2-methyl-n-1-tridecene), and reducing ethylene production by ACC deamination, solubilization of insoluble phosphate and other nutrients, nitrogen fixation (Bhattacharyya et al., 2012, 2014; Cassán et al. 2014; Park et al., 2015). Among those phytohormones, the bacterial IAA likely plays a central role (Spaepen et al. 2008) and it becomes the main trait in PGPR screening in most of studies. Secondly, the mechanisms involve biological control capacities, such as the induction induced systemic resistance (ISR) via jasmonic acid (JA) and ethylene (ET) signaling pathways, the synthesis of extracellular enzymes to hydrolyze the fungal cell wall (i.g. b-1,3-glucanase, chitinase), production of antibiotic and siderophores for iron chelation, or competition for niches in rhizosphere. The mode of actions also includes the stimulation of mycorrhizae development, or removal of heavy metal contamination or phytotoxic substances (Henry et al., 2012, 2013; P. Bhattacharyya & Jha 2012; Duca et al. 2014). The performance of PGPR on promoting plant growth, however, still relies on several factors, such as plant genotype, soil type, environmental conditions (temperature and humidity), microflora community (Bashan et al., 2014; Calvo et al. 2014).

Besides, PGPR were also able to increase the abiotic tolerance (i.g., salinity, drough, and heat stress) through reducing the production of 1-aminocyclopropane-1-carboxylic acid (ACC) (Calvo et al. 2014). For example, rhizosphere bacteria such as *Azospirillum*, *Bacillus*, *Rhizobium* and *Enterobacter* are able to reduce the production of the plant stress hormone ethylene via the secretion of 1-amino cyclopropane-1-carboxylate deaminase, thus preventing plant growth inhibition (P. Bhattacharyya & Jha 2012). Futhermore, PGPR can reduce the pollutant impacts, such as herbicides, pesticides, and heavy metal detoxification (Bhattacharyya & Jha 2012; Bashan et al. 2014; Upadhyay & Singh 2015).

Some PGPR-based biostimulants also have a biocontrol activity enabling them to induce plant resistance to various bioaggressors (Henry et al., 2013; P. Bhattacharyya & Jha 2012). Some of PGPR strains are able to emit microbe-associated molecular patterns (MAMPs) and trigger induced systemic resistance (ISR) in the host plant through jasmonic acid (JA) and ethylene (ET) signaling pathways, which elicit plant defenses (Géraldine et al, 2016). The MAMPs were best characterized with several species of *Pseudomonas* and *Bacillus* which are able to induce ISR and against a broad range of diseases caused by viruses, bacteria and

fungi (Lugtenberg et al., 2009). The MAMPs which act as elicitors of systemic resistance include flagellin (Meziane et al., 2005), lipopolysaccharides (LPS) (Tang et al., 2005), pyoverdines (Budzikiewicz, 2004), tri-N-alkylated benzylamine derivative (NABD) (Ongena et al., 2005), pyocyanine and 2,4-diacetylphloroglucinol (DAPG) (Iavicoli et al., 2003), fengycins and biosurfactants such as rhamnolipids and lipopeptides (Tran et al. 2007, Ongena et al., 2007), and volatile organic compounds (VOCs; i.g., 2,3-butendiol) (Ryu et al., 2004). Therefore, many PGPR strains were commercialized and registered as biocontrol products (Copping, 2009). However, this Ph.D. thesis mainly focusses on evaluating the impacts of PGPR on promotiong the plant growth and nutrient uptake rather than studying the capacities to improve biotic stress tolerance.

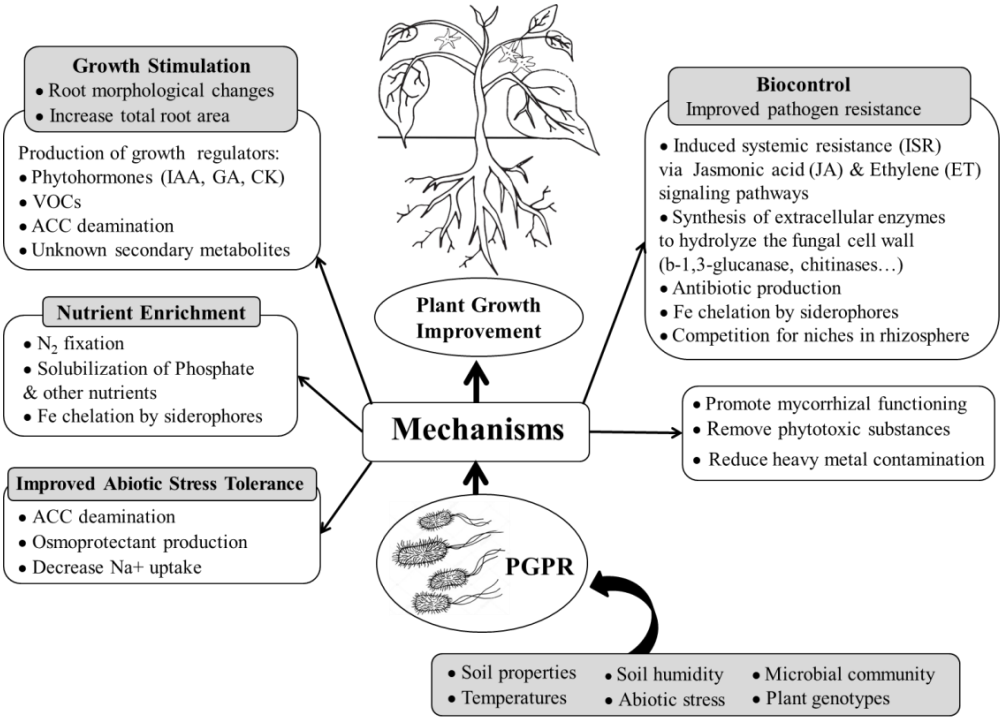

Figure 2. The mechanisms of PGPR used for plant growth stimulation and biological control improvement [based on the studies of Vacheron et al. (2013) and Vejan et al. (2016)]. The mechanisms invole the stimulation of plant growth, root morphological modification, and the enhancement of nutrient uptake, such as production of phytohormones (auxin (IAA), cytokinins, gibberellins), volatile organic compounds (VOCs), and decrease ethylene production by ACC deamination, solubilization of insoluble phosphate and other nutrients, N_2 fixation and increase the abiotic tress tolerance. The mechanisms for biocontrol involving the induction induced systemic resistance (ISR) via jasmonic acid (JA) and ethylene (ET) signaling pathways, the synthesis of extracellular enzymes to hydrolyze the fungal cell wall, production of antibiotic and siderophores for iron chelation, or competition for niches in rhizosphere, the stimulation of mycorrhizae development, or removal of heavy metal contamination or phytotoxic substances. The performance of PGPR on promoting plant growth depends on several environmental factors, such as soil type, soil temperature and humidity, microflora community and plant genotype.

1.1.2.2 Rhizobacterial IAA and cross-talking of hormonal pathways

The plant hormone auxin, naturally occurring auxin molecule as indole-3-acetic acid (IAA), is a key coordinating signal in many aspects of plant development. The basic function of auxin is to affect different plant processes such as stimulation of plant cell division and differentiation, initiation of lateral and adventitious roots, stem and root elongation (Teale et al., 2006).

The rhizobacterial IAA likely plays a central role in morphological changes in roots (Spaepen et al. 2008) and it becomes one of the key traits in PGPR screening and will be assessed in this study. A majority of the PGPR are able to produce indole-3-acetic acid (IAA) which is the most abundant form of auxin produced by PGPR. Most of PGPR use tryptophan as a precursor for IAA biosynthesis. Four different IAA-biosynthesis pathways have been described, in which three tryptophan-dependent pathways via indole-3-pyruvate (IPyA), indole acetamide (IAM), tryptamine (TAM) and one tryptophan-independent pathway (Prinsen et al. 1993; Carreño-López et al. 2000; Spaepen and Vanderleyden 2011) (Figure 3).

Besides IAA, they also produce other auxin-like molecules such as indole-3-butyric acid (IBA) and phenylacetic acid (PAA). Many other indolic compounds which could serve as precursors or storage compounds for IAA biosynthesis have been identified in the supernatants of *Azospirillum* sp., such as indole-3-lactic acid (ILA), indole-3-ethanol and indole-3-methanol, 3-acetamide (IAM), indole-3-acetaldehyde, tryptamine (TAM) (Spaepen and Vanderleyden 2011; Cassán et al. 2014).

The reports of the role of rhizobacterial auxin in regulating the plant-microbe interactions are still incomplete. Several recent reviews summarize many aspects of auxin biosynthesis, transport and auxin signaling pathway in root morphological changes induced by PGPR (Zhao 2010, Sukumar et al., 2012) (Figure 4)

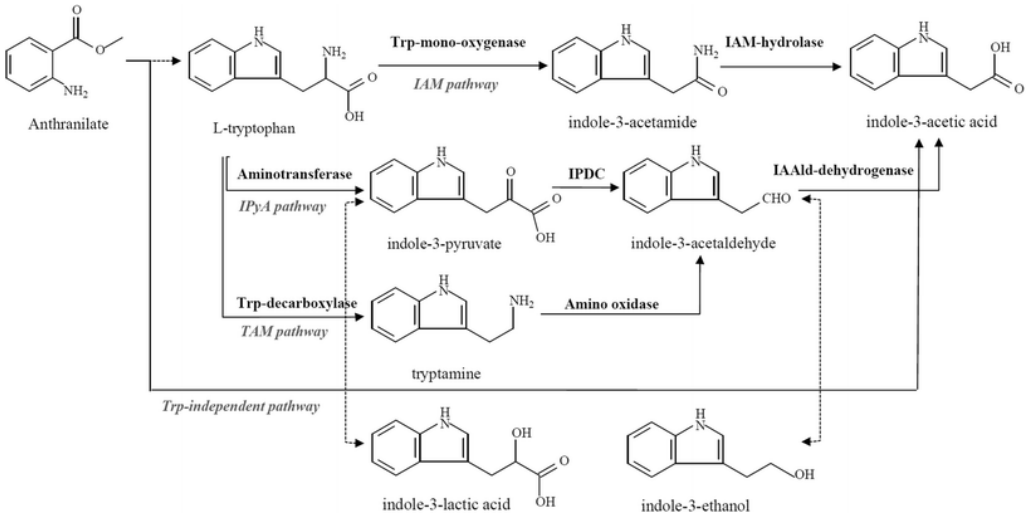

Figure 3: Overview of rhizobacteral indole-3-acetic acid (IAA) biosynthetic pathways in *Azospirillum*. Dashed lines represent hypothetical conversion steps to storage products. Trp, tryptophan; IPDC, indole-3-pyruvate decarboxylase; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide (Cassán et al. 2014)

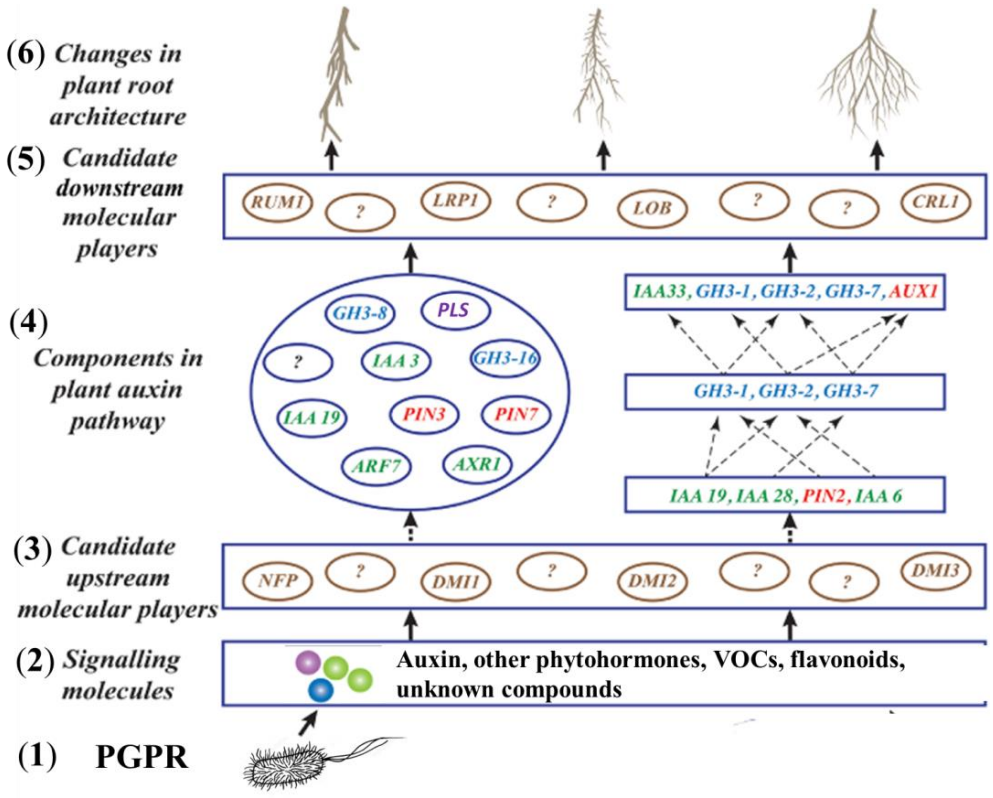

Figure 4. Hypothetical and simplified model of auxin signaling pathway modulated by PGPR in order to alter root morphology, as suggested by Sukumar et al. (2012). The model is described as follows: **(1) PGPR**; **(2) Signalling molecules**: different color circles represent different potential signaling molecules such as auxin (IAA), other auxin-like molecules, other phytohormones, VOCs, flavonoids and proteins; **(3) Candidate upstream molecular players**: potential proteins involved in altering root morphology, such as doesn't make infections (DMIs, related to nodulation and mycorrhiza formation), Nod factor perception (NFP); **(4) Components in plant auxin pathway**: auxin transport proteins (PINs and AUX1), auxin signaling proteins (IAAs and ARFs), auxin conjugation (GH3s); POLARIS (PLS); proteins in circles are temporally undetermined, proteins in boxes are temporally determined, dotted arrows represent potentially related network, the question marks represent unknown proteins and factors; **(5) Candidate downstream molecular players**: potential proteins regulating root morphological modifications, such as crown rootless (CRL1), lateral organ boundaries (LOBs), lateral root primordium 1 (LRP1), rootless with undetectable meristem 1 (RUM1); **(6) Changes in plant root architecture**: different root morphological changes resulting from different auxin signaling pathways induced by PGPR.

The cross-talking between bacterial IAA with other the phytohormones (such as cytokinins, gibberellins, ethylene…) or VOCs produced by PGPR which lead to their plant growth-promoting activity have been shown in previous studies, particularly in the well-studied PGPR genus *Azospirillum* (Cassán et al. 2014). The balance between auxin and cytokinins (CKs) in plants which depends on their ratio of production/degradation modulates the root/shoot ratio. However, there has been no study on the effect of rhizobacteria on this IAA/CKs balance. Several studies showed a cross-talking between IAA and gibberellins (GAs). Several PGPR are able to produce GAs (Esquivel-Cote et al., 2010). The improvement of plant growth induced by PGPR could be partly due to the impact of GAs produced by PGPR or the increase of plant production of GAs induced by rhizobacterial IAA (Yaxley et al., 2001; Ford et al., 2002).

The level of ethylene can be altered by rhizobacterial IAA in inoculated plants. Krumpholz et al. (2006) figured out a positive correlation between IAA and ethylene levels in the tomato seedling inoculated with an IAA-producing *A. brasilense* strain FT326, in which the levels of IAA and ethylene were higher in inoculated plants. Such positive correlation between IAA and ethylene level resulted in enhancing plant biomass, number and length of main roots. This suggested a cross-talking between rhizobacterial IAA and plant ethylene biosynthesis (Rahman et al. 2002). IAA can stimulate the ethylene biosynthesis through transcriptionally activating genes coding for 1-aminocyclopropane-1-carboxylate synthase (ACS), which controls the limiting step of ethylene biosynthesis (Kende 1993).

There is an interaction between auxin signalling pathway involved in PGPR-plant interaction with several volatile organic compounds (VOCs) emited by PGPR. The auxin signalling pathway related to PGPR response can be induced without IAA production of PGPR. Zhang et al. (2007) demonstrated that volatile organic compounds (VOCs) produced by *B. velezensis* GB03 were able to promote plant growth in *Arabidopsis* by regulating auxin homeostasis in host plants. Nitric oxide (NO) is a volatile participating in signaling of defense and developmental pathways in plants (Lamattina and Polacco, 2007), and it is produced by several PGPR strains (Molina-Favero et al. 2008). Correa-Aragunde et al. (2006) demonstrated that NO plays a central role as an intermediate of the IAA signaling pathway in roots and results in lateral and adventitious root formation.

1.1.3 Impact of PGPR on nutrient enrichment and nutrient uptake efficiency

Integrated nutrient management is one of the current tools of agroecology. It does not imply the complete removal of chemical fertilizers from the agricultural production system. It actually promotes the use of alternative strategies to reduce their use (Wezel et al., 2014). Integrated nutrient management could employ PGPR to boost (i) *nutrient enrichment* in soil and/or (ii) *nutrient uptake efficiency* in order to enhance the crop productivity while it helps to reduce the negative impacts of mineral N, P fertilizers.

(i) PGPR-enriched nutrient availability

The nutrient availability in soil can be improved by PGPR implementation. With regards to increased nutrient availability, two main types of bacterial activities can be considered, that are nitrogen (N_2) fixation and nutrient solubilization (P. Bhattacharyya & Jha 2012; Calvo et al. 2014). N and P are the most limiting nutrients in soil for the crop growth (Schachtman et al., 1998), and supplying N and P fertilizers is therefore necessary to obtain the maximum potential of crop yield.

Some PGPR strains are able to fix N_2 in the atmosphere and solubilize insoluble phosphate in soil. For instance, the PGPRs belonging to *Rhizobium* are able to fix N_2 in nodules of legume plants and establish symbiotic interactions with legume crops, which have been extensively studied (Terpolilli et al., 2012). Beside of the symbioses with legumes of nodule-forming rhizobia, there are several PGPR belonging to non-legume N_2-fixing rhizobacteria, which associate with other host crops (Rai and Greene, 2006). For example, PGPR belonging to genera *Azospirillum* are able to increase yield of various crops, such as wheat, rice, sugar cane, cotton (Bashan et al. 1997, 1998, 2014). However, the plant growth-promoting capacity of such PGPR in non-legume plants does not seem to be due to their N_2–fixation ability but rather to other mechanisms such as improving root growth and nutrient uptake (Adesemoye et al. 2010).

Besides N_2-fixation, other PGPR strains are able to solubilize insoluble phosphate in soil. Phosphorus is the second most important nutrient for crops, just after N. However, it is less mobile compared with N and other nutrients, and it is often present as relatively unavailable forms (in metal complexes of both organic and inorganic pools) for the uptake of crops. The high reactivity of P with several metals (such as Fe, Al, and Ca) leads to the precipitation of 75–90% of P in the soil (Gyaneshwar et al. 2002). Fortunately, there are some PGPR that can liberate phosphate from the pools of insoluble phosphate due to their excretion of organic

acids and phytases. This makes phophorus available for the uptake by the roots (Richardson et al., 1994, 2001).

Several bacterial genes responsible for phosphate solubilization and phosphate uptake are induced by phosphate starvation, such as *pst* (Pi-specific transporter), *phoA* (alkaline phosphatase), *glpQ* (glycerophosphoryldiester phosphodiesterase), *phyC* (phytase), and *ushA* (nucleotidase) (Ishige et al, 2003; Prágai et al., 2004). However, more studies are needed to identify the molecules related to mode-of-action regulating mineral phosphate solubilization by PGPR. The uptake of phophorus from soil and transfer to plant tissues are mediated by a phosphate transporter (PT). Several phosphate transporter genes were found in wheat (Tian et al., 2017), but most of them were studied in the context of plant–*Arbuscular mycorrhiza* fungi interaction. Liu et al. (2018) found that the relative transcript levels of a phosphate transporter gene TaPT4 in wheat were lower in roots inoculated with *Pseudomonas* sp. strain P34-L. However, the mechanism is still unclear.

(ii) PGPR-enhanced nutrient uptake efficiency (NUE)

The term of nutrient use efficiency is often used deceptively and inconsistently in many previous studies (McDonald et al, 2014). The definition which is commonly used was proposed by Moll et al. (1982), in which the term nutrient use efficiency was originally used for nitrogen use efficiency. It has subsequently been extended for other nutrients. Nutrient use efficiency is defined as the yield of a crop per unit of nutrient supplied. It has two components: the ability to uptake the nutrients from the soil (so-called *nutrient uptake efficiency*) and the ability to convert the nutrients uptaken by the crop into grain yield (so-called *nutrient utilization efficiency*). In our study, only the term of *nutrient uptake efficiency* is used while the term of *nutrient utilization efficiency* was not employed, since the plants are harvested prior to the forming of ripe grains. Finally, we use only the term of *nutrient uptake efficiency* (NUE), and the NUE of a nutrient element is calculated as the total nutrient amount (g or mg) in plant per total nutrient supplied (g or mg) of that element, in which the total nutrient supplied is the sum of applied fertilizer and the available amount of that element in soil (Moll et al., 1982). The NUE in crops is often low and less than half (10-40%) of the applied fertilizer in the field is effectively absorbed by plants, while 60-90% of chemical fertilizers are generally lost by leaching, erosion, phosphorus precipitation, and volatilization (Adesemoye & Kloepper 2009). This results in serious environmental problems such as eutrophication of water resource

and greenhouse-gas emissions (Chien et al. 2011). Therefore, among the alternatives to improve the mineral fertilizer use efficiency, PGPR have been proposed as an effective agro-ecological solution for such environmental issues (Calvo et al. 2014; Le Mire et al. 2016). Several previous studies reported that PGPR are able to increase total nutrient uptake as well as the nutrient uptake efficiency (NUE) of N and P (an increase of approximately 2–4% compared to the NUE of the non-inoculated control) in wheat plants (Spaepen et al. 2008; Ahmad et al. 2017).

It has been demonstrated that the increase in root development as well as total root surface by inoculation with PGPR is a key tool to increase nutrient uptake and fertilizer use efficiency (Adesemoye & Kloepper 2009). The mode of action for such morphological changes in roots are mainly due to their capacity to produce/degrade various plant-growth regulators, i.e. phytohormones or analogs produced by PGPR, such as indole-3-acetic acid (IAA), cytokinins, gibberellins, volatile organic compounds (VOCs), and reducing ethylene production by ACC deamination (Duca et al. 2014). Such plant-growth regulators produced by PGPR can modulate the hormonal balance in the host plants and regulate multiple plant physiological processes, including root initiation and elongation, root hair formation (Calvo et al. 2014). Among those phytohormones, the bacterial IAA likely plays a central role (Spaepen et al. 2008) and it becomes the main trait in PGPR screening and will be assessed in this study.

Recent studies have shown that PGPR could be employed to reduce the use of high mineral N fertilizer dose (Dobbelaere et al., 2002; Adesemoye et al. 2009). Dobbelaere et al. (2002) found that the inoculation of wheat plants with *A. brasilense* Sp245 could reduce by up to 26% the fertiliser dose without affecting the yield. Adesemoye et al. (2009) showed that a combined inoculation of the two PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4 with a strain of the arbuscular mycorrhizal fungus *Glomus intraradices* reduced fertilizer use by 25%. This combination was as efficient as 100% fertilizer application in terms of plant growth, yield, and nutrient uptake. Other examples of PGPR-based biostimulants that enhance crop growth and reduce the amount of needed chemical fertilizers are given in Table 1.

Table 1. Examples of promising PGPR-based biostimulants for the improvement of nutrient uptake and plant productivity, or reduction of chemical fertilizers under various experimental conditions.

Biostimulant	**Enhancement of crop growth and reduction of chemical fertilizer level**	**Crop**	**Experimental conditions**	**References**
Bacillus megaterium M3, *Bacillus* OSU-142, *Azospirillum brasilense* Sp.245, *Paenibacillus polymyxa* RC05, *Bacillus megaterium* RC07, *Bacillus licheniformis* RC08, *Raoutella terrigena*, *Burkholderia cepacia* FS Tur	Plant root and shoot weight increase under greenhouse conditions. Single and combinations of PGPR increased yield up to 40.4% for wheat and 33.7% for barley under field conditions and in combination with N fertilizer.	Wheat Barley	Greenhouse and field	(Çakmakçi et al. 2014)
Pseudomonas aeruginosa	Improved N and P uptake. Increase in leaf chlorophyll amounts and plant biomass under Zn stress (enhancement of antioxidant enzymes, ascorbic acid and total phenolics)	Wheat	Greenhouse	(Islam et al. 2014)
Arthrobacter sp. and *Bacillus subtilis*	Increased plant tolerance to salinity. Plant dry weight increased up to 26% and 40% under 2 $dS.m^{-1}$ and 6 $dS.m^{-1}$ salinity level, respectively.	Wheat	Greenhouse	(Upadhyay & Singh 2015)

Burkholderia vietnamiensis AR112	Increased or equivalent weight and yield of traditional rice compared with 100% N chemical fertilization.	Rice	Field	(A.E.D.S. Araújo et al. 2013)
Bradyrhizobium spp. and concentrated metabolites from *Bradyrhizobium diazoefficiens*	Increased grain yield by 4.8% compared with the exclusive use of *Bradyrhizobium* spp.	Soybean	Greenhouse and field	(Marks et al. 2013)
Rhizobium tropici CIAT899, *Glomus intraradices*	Increase in P and N amounts up to 40% and 42%, respectively, in soil. Nodule number enhanced by 70% and nodule mass by 43%. Plant shoot dry weight increased by up to 24% and root growth by up to 48%.	Bean	Greenhouse	(Tajini et al. 2012)
Pseudomonas jessenii, *Pseudomonas synxantha* and a local AM	PGPR or AMF alone increased yield by up to 29% and 31%, respectively. Combining PGPR and AMF increased the yield by up to 41%.	Wheat	Field	(Mäder et al. 2011)
Bacillus amyloliquefaciens IN937a, *Bacillus pumilus* T4, *Glomus intraradices*	Inoculation of PGPR and AM together reduced fertilizer use by 25% Combination was equivalent to 100% fertilizer application for plant growth, yield and nutrient uptake.	Tomato	Greenhouse	(Adesemoye et al. 2009)
Bacillus subtilis	Plant growth and yield enhanced by up to 30% compared with NPK fertilization.	Cotton	Field	(Yao, H. Bochow, et al. 2006)

1.2 Implementation challenges in agricultural practices

The literature supports the implementation of PGPR-based biostimulant tools in agroecological practices, with clear demonstrations of their potential to reduce chemical inputs, save energy and provide farmers with new opportunities for sustainable fertilization and disease control (Calvo et al. 2014). The agroecological use of these tools will obviously require a shift in conventional practices from total reliance on fertilizers to the integrated management of biotic and abiotic stresses (Wezel et al. 2014). However, biostimulant products are not yet used as routine tools in agriculture. In the second half of this review, we explain the drawbacks restricting the widespread use of PGPR in agriculture, and what is being developed to enhance their use, and thus make an important contribution to the agroecological and sustainable management of cultivated ecosystems.

1.2.1 Screening biostimulant products

The screening of suitable PGPR inoculants for specific crops, growth conditions and pathogens is critical if the efficacy of these products in the field is to be guaranteed. A common method for screening an effective PGPR inoculant is to isolate strains from plant growth-promoting soil or from pathogen-suppressive soil (Mendes et al. 2011). Screening failures can occur, as some PGPR strains which show limited ability to promote plant growth during screening trials under controlled conditions can be among the most effective strains in the field (Araújo et al., 2013). Such results could be due to the differences between field and controlled conditions, and it appears that PGPR, which increase the nutrient uptake capacity of roots in a large field, cannot express such properties easily *in vitro* or in small-scale pots. In addition, the screening of multiple microbes in consortia is complicated and requires considerable time and research, as well as knowledge of microbial ecology and of the interactions between the strains of a biostimulant product, the host plant and the local rhizomicrobial community. Recent progress in molecular biology and biotechnology will probably facilitate PGPR screening (P. Bhattacharyya & Jha 2012).

1.2.2 Formulation and application methods

The formulation and application method are probably among the most critical parameters determining the efficiency of biostimulant products. The formulation must maintain an effective plant-growth promotion or biocontrol capacity and be

easy to use (Bashan et al. 2014). In the case of PGPR inoculants, Bashan et al. (2014) summarized various formulation methods, from the choice of carriers (peat, coir dust, charcoal, sawdust, clay, perlite, vermiculite, polymer-like alginate) to the formulation process. They also summarized various practical techniques for inoculant application and production achievement. Seed treatment has attracted attention as a simple and economically viable technique, being convenient for both farmers and industry (Bashan et al., 2014). The seeds are usually coated with a carrier and PGPR, with or without adhesives (carboxymethyl cellulose, sucrose, vegetable oil, Arabic gum). This is currently the method most often used to apply PGPR inoculants as it ensures an optimal threshold number of PGPR cells per seed needed to cover the seedling roots. Although the cell threshold differs among strains, the common concentration is 10^8 cells per plant (P. Bhattacharyya & Jha 2012). Soil applications of PGPR are performed when a large population of rhizobacteria is needed at a specific and crucial plant growth stage (*e.g.* tillering or flowering stages) (Bashan et al., 2014). However, soil and open-air conditions (humidity, temperature) can affect the success of the soil application. Extreme temperatures can cause a decline in the PGPR survival rate, and soil humidity determines the effective mobility of the inoculated bacteria in the rhizosphere (Bashan et al. 2014). Using enough water (*e.g.* at least 400L ha^{-1}, according to the manufacturers' use instruction) in the mixture with liquid or powder-based inoculants also ensures that the bacteria are positioned near the root system. Additional PGPR inoculations could be needed to maintain a minimal bacteria population in the case of stressful conditions such as winter and drought (Bashan et al. 2014).

1.2.3 PGPR implementation under greenhouse and field conditions

Biostimulant products can be based on a single PGPR strain, a PGPR mix or a mix of PGPR and PGPF. There is growing scientific evidence supporting the use of PGPR inoculants as biofertilizers for many plants under greenhouse and field conditions. Compared with single strain products, consortia can reach most of the empty niches because of their increased genetic diversity and they colonize the root zone much faster than single strains (Reddy, 2014). Products with a mix of PGPR strains can therefore compete spatially with a broader range of potential pathogens under different plant growth and environmental conditions (Reddy 2014). For example, FZB24® was shown to promote plant growth and yield in cotton, tomato and maize (Kilian et al. 2000a; Yao, H. Bochow, et al. 2006). Similarly, RhizoVital42® proved efficient in lettuce (Chowdhury et al. 2013; Kröber et al. 2014), BactofilA10® in rye-grass (Tállai et al. 2012), and TwinN® in sugarcane

(Simwinga et al. 2010). Further information on commercialized PGPR products is given in Table 2.

Products containing exogenous PGPR compounds (*e.g.* exopolysaccharides, phytohormones) have also been developed to enhance the growth of specific beneficial microbes in the soil (Marks et al. 2013). The efficiency of PGPR-based products, however, still relies on several factors. Plant species and variety (releasing different types of root exudates), soil type, environmental conditions, and the commercial formulation are crucial determinants of the efficient and reproducible action of inoculated PGPR (Calvo et al. 2014). The best PGPR products generally consist of local strains that are specific to the host plant, show good capacity for physiological and genetic adaptation and co-evolve with other native strains in a common habitat (Reddy et al. 1999; Mäder et al. 2011; A.E.D.S. Araújo et al. 2013).

Besides, the success of bacterial inoculation is influenced by complex environmental factors, such as soil properties, soil temperature and humidity, microbial community, plant host variety, and farming practices. In other words, the plant growth-promoting capacity of PGPR will perform differently from this growth condition to another condition. For example, the plant growth-promoting effect of PGPR have been proven to depend on the rate of mineral N fertilization (Shaharoona et al. 2008; Veresoglou & Menexes 2010; Hussain et al. 2016; Spolaor et al. 2016). Therefore, it is always questionable whether the positive results on plant growth induced by PGPR under *in vitro* conditions can be reproduced under greenhouse conditions and then under field conditions (Veresoglou & Menexes 2010, Parnell et al., 2016).

Table 2 Examples of commercial PGPR-based products in Europe, North America and Asia.

Products	**Rhizobacteria**	**Crop**	**Manufacturer**
Amase®	*Pseudomonas azotoformans*	Cucumber, lettuce , tomato, pepper	Lantmannen Bioagri, SWEDEN
AmniteA100®	*Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Chaetomium*		Cleveland Biotech, UK
BactoFil A10®	*Azospirillum brasilense*, *Azotobacter vinelandii*, *B. megaterium*, *B.polymyxa*, *P. fluorescens*	Monocotyledons (cereals)	AGRO.bio Hungary Kft., HUNGARY
BactoFil B10®	*Azospirillum lipoferum*, *Azotobacter vinelandii*, *B. megaterium*, *B. circulans*, *B. subtilis*, *P. fluorescens*	Dicotyledons (sunflower, potato, rapeseed)	
Cérès®	*Pseudomonas fluorescens*	Field and horticultural crops	Biovitis, FRANCE
Compete ® Plus	*B. azotofixans*, *B. licheniformis*, *B. megaterium*, *B. polymyxa*, *B. pumilus*, *B. subtilis*	Field crops, tree nurseries	Plant Health Care, USA
FZB24®fl	*B. amyloliquefaciens ssp. plantarum*	Ornamentals, vegetables	ABiTEP GmbH, GERMANY
Rhizovital 42®	*B. amyloliquefaciens*	Field crops	
Gmax® PGPR	*Azotobacter*, *Phosphobacteria*, *P. fluorescens*	Field crops	Greenmax AgroTech, INDIA
Inómix® Biostimulant	*B. polymyxa* (IAB/BP/01), *B. subtilis* (IAB/BS/F1)	Cereals	IAB (Iabiotec), SPAIN

Inómix® Biofertilisant	*B. megaterium, Saccharomyces cerevisiae, Azotobacter vinelandii, Rhizobium leguminosarum*	Cereals	IAB (Iabiotec), SPAIN
Inómix® phosphore	*P. fluorescens, B. megaterium , Saccharomyces cerevisiae*		
Micosat F® Uno	*Agrobacterium radiobacter AR 39, B. subtilis BA 41, Streptomyces spp. SB 14*	Fruits, vegetables, flowers	CCS Aosta Srl, ITALY
Micosat F® Cereali	*B. subtilis BR 62, Paenibacillus durus PD 76, Streptomyces spp. ST 60*	Cereals, tomatoes, sunflowers, beet, soybeans	
Nitroguard®	*Azospirillum brasilense NAB317, Azorhizobium caulinodens NAB 38, Azoarcus indigens NAB04, Bacillus* sp.	Cereals, seed rape, sugar beet, sugarcane, vegetables	Mapleton AgriBiotec Pty Ltd, AUSTRALIA
TwinN®	*Azospirillum brasilense NAB317, Azorhizobium caulinodens NAB 38, Azoarcus indigens NAB04*		
PGA®	*Bacillus* sp.	Fruits, vegetables	Organica technologies, USA
Rhizocell ® GC	*B. amyloliquefaciens* souche IT45	Cereals	Lallemand Plant Care, CANADA
Symbion®-N	*Azospirillum, Rhizobium, Acetobacter, Azotobacter*	Field crops, vegetables	T.Stanes & Company Ltd, INDIA
Symbion®-P	*B. megaterium var. phosphaticum*		
Symbion®-K	*Frateuria aurantia*		

1.2.4 Farmers and the use of alternative methods

Farmers do not always greet the suggestion of using alternative methods with much enthusiasm, especially those on small-scale farms or in developing countries (Gozzo & Faoro 2013; Bashan et al. 2014). They tend not to adopt biostimulant products or use strategies unless their success is guaranteed. The highest number of farmers currently using plant biopesticides, is in North America, representing 40% of the agri-market, compared with 25% in Europe, 20% in Asia, 10% in South America and 5% in the rest of world (Cox & Wong 2013).

The main reason for farmer skepticism about these alternative methods relates to their variable efficacy in the field compared to conventional chemical inputs (Arora et al. 2010; Walters et al. 2013). Many studies have shown that these products can have a variable field performance, in contrast to the promising results obtained in the laboratory or in greenhouse conditions (Gozzo & Faoro 2013). There are several reasons for this inconsistency in practical conditions.

In the case of PGPR, bacteria concentrations in commercialized products can fall below the desired threshold (usual concentration: 10^8-10^{11} cells·ml^{-1}), especially under long-term or inadequate storage (Bashan et al. 2014). A less effective interaction can also occur when the PGPR inoculant is not adapted to the host plant or the local environmental conditions (climate, soil characteristics, agronomic practices) and crop systems (plant genotype, nutritional requirements, physiological stage) and the formulation. For example, modern rice varieties selected to use N fertilizers effectively are less interactive with native N-fixing bacteria than traditional varieties (Araújo et al., 2013).

Farmers' decisions on whether or not to adopt new methods often depend on how much they want to change their agricultural practices. Total reliance on new strategies can be challenging. The benefits of these strategies have to be clearly demonstrated through educational programs that focus on field data (*e.g.* pest/disease identification, timing of infestation, crops) (Rodriguez-Saona & Stelinski 2009). This includes detailed knowledge about agronomic parameters and designing adapted crop management techniques, with the appropriate biostimulant product applied at the right time and frequency, in combination with other control methods and on responsive cultivars (Walters et al. 2013; Bashan et al. 2014).

Henceforth, tools need to be designed that meet farmers' demands by ensuring: optimal crop yield with lower input costs; compatibility between the applied products and soil conditions, farming machines and equipment; and good shelf life and long-term survival during storage, especially with PGPR inoculants (Bashan et

al. 2014). The use of biostimulant products into agricultural practices depends on their economic relevance compared to conventional practices (Rodriguez-Saona et al. 2009; Bashan et al. 2014). Further research is needed on improving the understanding of which field conditions are most suited to the use of a specific biostimulant product (P. Bhattacharyya & Jha 2012). Scientists are aware of the stakes involved here and many partnerships have been launched. A European stakeholders association, the European Biostimulants Industry Council (EBIC), was founded in 2011 and provides technical and practical information on plant biostimulants (Traon et al. 2014).

1.2.5 Regulatory framework

A large number of biostimulant products have long been known and have been patented for agricultural plant growth-promotion and/or pest management but they are still not available commercially in the EU, unlike the situation in other countries in the world (Dayan et al. 2009). Many products that encourage plant growth or plant protection have not been registered and there is a lack of fit-for-purpose regulatory procedures in the EU because of the time and costs of registration (Arora et al. 2010; Köhl 2010; Walters et al. 2014). The approval of any Plant Protection Product (PPP) requires the registration of the active material on a list validated by the EU (European Parliament 2009a).

In the case of biostimulants, these products are still not covered by EU regulatory procedures and need to be included in the framework of PPP products or placed on the market following national fertilizers laws. The Fertilizers Regulation 2003/2003 covers only the placing of inorganic fertilizers (EC fertilisers) on the market and has been under revision since 2012-2013, with the aim of extending its scope to other fertilizing and related materials, such as plant biostimulants and fertilizer additives (Traon et al. 2014).

The current strategy of the EU in sustaining the development of new biostimulant methods in agriculture is implemented *via* various legislative procedures. Regulation 1107/2009 aims to harmonize the overall procedures authorizing plant protection products in the EU market. It also facilitates the approval of natural substances (Article 23), thereby simplifying the regulation procedures for natural preparations with low risk. The EU has proposed granting the first approvals for agrochemicals in a new category entitled ‘basic substances’(European Parliament 2009b). The reduction of conventional inputs is also planned in other European countries, including Belgium (NAPAN, 2013), Germany (National Action Plan on Sustainable Use of Plant Protection Products, 2013) and the UK (UK NAP, 2013). The promise of strong growth in the biostimulant market in a near future has also led major

agrochemical companies to invest in these green technologies. All stakeholders in the agricultural sector, including agricultural distributors and plant breeders, could play an important role in promoting the use of biostimulant products.

1.3 Conclusion and perspectives

Strong efforts are being made to improve attitudes in the farming community, and in society in general, towards the use of alternative methods to chemical inputs. It is widely agreed that PGPR biostimulants should not be used as stand-alone methods in agroecological management, but integrated into fertilization and disease/pest control strategies to complement chemical inputs and contribute to a reduction in their dosage amounts and application frequency. Although these tools have been widely endorsed for their advantages, farmers and growers are still not completely confident about using them, mainly because of their fluctuating field performance (Beckers & Conrath 2007, Bhattacharyya & Jha 2012). Farmers need more information on how to use these tools in their agricultural practices. Regulators, investors, growers and consumers also need to be well informed about the advantages of these alternative methods and their potential in promoting sustainable agriculture.

Further research is needed to better understand the environmental parameters affecting the efficiency of these products, particularly for field crops. Special attention should also be given to the formulation and the potential interactions of these products with the plant environment. Multidisciplinary research groups, such as the AgricultureIsLife platform (Gembloux Agro-BioTech, Université de Liège, Belgium), should address the question of how best to use these tools, given current practices, by studying the issues that still need to be overcome (*e.g.* screening methodology, formulation, environmental impact).

Many challenges remain before biostimulant products can be widely and successfully used on a commercial basis, but the intensive efforts in research and the legislative area, as well as in enhancing society's awareness of these products, will increase their credibility and acceptance (Wezel et al. 2014). Agricultural practices using these tools need to be adapted (*e.g.* using cultivars specifically chosen for the appropriate responses) (Walters et al. 2014). In Europe, the long-term objective to be pesticide free is already leading to changes in crop management practices and represents a major driver in the use of biostimulant products. Within the context of climate change, increasing environmental concerns and population increase, these

alternative methods offer important potential tools for achieving sustainable food production.

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2

Thesis Objectives

2 Thesis Objectives

The objectives of this PhD project are to (i) select PGPR strains to enhance the wheat growth and productivity, (ii) evaluate the impact of rhizobacterial strains on the nutrient uptake efficiency, and (iii) assess the impact of environmental factors on the plant-PGPR interaction. In this PhD project, PGPR-based biostimulants have been collected and screened for their plant growth promoting capacity under gnotobiotic, greenhouse, and field conditions.

Plant growth-promoting rhizobacteria (PGPR), which belongs to microbial biostimulants, are able to enhance root growth, nutrient uptake as well as fertilizer use efficiency in plants. The fertilizer use efficiency of crops is often low due to the large portion of the applied fertilizer generally lost by leaching, erosion, P precipitation, and N volatilization (Adesemoye & Kloepper 2009). Therefore, among the alternatives to improve the mineral fertilizer use efficiency, PGPR have been proposed as an effective agro-ecological tool to reduce the use of chemical fertilizer and increase nutrient uptake efficiency (NUE) (Le Mire et al. 2016). However, the success of PGPR inoculation is influenced by complex environmental factors, such as the rate of mineral N fertilization (Veresoglou & Menexes 2010; Spolaor et al. 2016). Therefore, the first two questions focus on the impact of mineral N fertilization levels on the performace of PGPR as well as the impact of PGPR on the nutrient upake of the inoculated plants:

Question (1): *what is the optimum N fertilization rate that would allow the PGPR strains to promote plant growth under in vitro, greenhouse, and field conditions?* It is hypothesized that the absence of N fertilizer supply could maximize the effect of PGPR on wheat growth as suggested by previous studies (Shaharoona et al. 2008; Veresoglou & Menexes 2010; Hussain et al. 2016; Spolaor et al. 2016).

Question (2): *Is there any relationship between the increased plant biomass caused by PGPR inoculation and the improvement of nutrient uptake in terms of nutrient concentration and total nutrient content?* The pronounced increase in biomass promoted by PGPR inoculation is hypothesized to go along with an increase of nutrient uptake in term of nutrient concentration in plants.

Screening PGPR strains according to the particular genotype of a crop is a critical step for the success of bacterial inoculation on promoting the plant growth. However, the responses to PGPR inoculation could be different according to the developmental stages of a crop. This might be due to the different rates of

rhizodeposition of roots at different plant growth stages. The third question therefore focusses on the interaction of PGPR response and the plant developmental stages:

Question (3): *Which developmental stages of wheat are more responsive to biostimulant effects of PGPR?*

Finally, it is always wondered if the positive results on plant growth induced by PGPR inoculation under gnotobiotic conditions can be reproduced under greenhouse conditions and later under field conditions (Veresoglou & Menexes 2010). To deal with this hypothesis the fourth question is addressed:

Question (4): *Is the plant growth-promoting effect of PGPR reproducible in different cultivation systems, i.e. from gnotobiotic, greenhouse, towards field conditions?*

Before answering those questions, a bibliographic study was conducted in order to have an overview of the implementation of PGPR-based biostimulants in the agroecological management of cultivated ecosystem (Chapter 1). The research questions and the aims of the thesis are shown in the present chapter (Chapter 2). The methodology that has been followed is explained in Chapter 3.

The answers to the question (1) can be found in Chapter 4 and 5 which study the interaction between different PGPR and different N rates under *in vitro*, greenhouse, and field conditions. The answers to the question (2) can be found in Chapter 4 and 5 which focuss on the effect of PGPR on nutrient uptake of plants grown under greenhouse conditions. The answers to the question (3) can be found in Chapter 4 which studies the effect of PGPR on plant growth at different developmental stages of wheat.

The answer to the questions (4) can be found in Chapter 5, which focusses on plants grown under *in vitro*, greenhouse and field conditions. To achieve these objectives, experiments were designed using several commercial PGPR strains with plant growth-promoting capacity in preliminary greenhouse experiments, then inoculated to spring/winter wheat cultivars under gnotobiotic or greenhouse conditions, and finally tested in field trials for winter wheat in a combination with different N fertilization schemes.

Chapter 6 presents the general discussion, conclusion, and perspectives. The scientific communications related to this PhD thesis and they are presented in chapter 7.

3

Strategic Choices

3 Strategic Choices

3.1 PGPR strains

The selection of PGPR strains is a key factor for the success of bacterial inoculation on crops. Among PGPR genera, *Bacillus, Azozpirillum, Azotobacter,* and *Sinorhizobium* have been well studied and commonly formulated into commercial products in agricultural market. They are able to associate with various cereal species (Hungria et al. 2010). Therefore, the strain selection mainly targets PGPR strains belonging to *Bacillus, Azozpirillum, Azotobacter, and Sinorhizobium* groups which likely have the capacities of improving root growth and nutrient uptake on wheat.

Several PGPR strains were collected. for preliminary screening based on the capacity of plant growth promotion under greenhouse conditions. The criteria for such a selection of PGPR strains were (i) the ability of PGPR to promote plant growth of wheat, barley or other cereals based on scientific reports or manufacturers' claims, and (ii) the concentration of bacterial cells or spores ($\geq 10^9$ CFU g^{-1}) in commercial products in order to facilitate the bacterial application in field trials (Table 3). 13 PGPR strains were selected for this purpose, including: (1) consortia of *Azospirillum brasilense* NAB317, *Azorhizobium caulinodans* NAB38, *Azoarcus indigens* NAB04, (2) *Bacillus velezensis* IT45 (formerly referred to as *B. amyloliquefaciens / subtilis* IT45; *Fan et al.*, 2017*)*, (3) *Bacillus velezensis* FZB24 (formerly referred to as *B. amyloliquefaciens / subtilis* FZB24; *Fan et al.*, 2017) , (4) *Bacillus velezensis* FZB42 (formerly referred to as *B. amyloliquefaciens / subtilis* FZB42; *Fan et al.*, 2017), (5) *Azospirillum brasilense*, (6) *Azotobacter chroococcum*, (7) *Azospirillum brasilense* SP245, (8) *Azospirillum brasilense* SpBr14, (9) *Azospirillum brasilense* 65B, (10) *Azotobacter chroococcum* AV, (11) *Bacillus megaterium* SNji, (12) *Sinorhizobium meliloti* L4, and (13) *Bacillus velezensis* GB03 (formerly referred to as *B. amyloliquefaciens / subtilis* GB03; *Fan et al.*, 2017). The bacteria were individually inoculated to spring wheat grains and then sown in a mixture of soil and sand (3 soil : 1 sand; w/w) without additional fertilizer. The plants were grown as described in the "Greenhouse experiments" section in Chapter 4. The plants were harvested after 30 days of growing at Zadoks stages 30-31.

The results of preliminary screening are shown in Figure 5. Based on the most significant increases in root, shoot and total dry biomass from preliminary

screenings in such greenhouse tests, the three best strains – *Bacillus velezensis* GB03 (BveGB03), *Bacillus megaterium* SNji (BmeSNji), and *Azospirillum brasilense* 65B (Abr65B) – were selected for the study of interaction between PGPR, wheat and mineral N fertilizer rates under gnotobiotic and greenhouse condition (Chapter 4). Besides, three other strains of *B. velezensis*, i.e. IT45 (Rhizocell GC, Ithec, Lallemand Plant Care SAS, France), FZB24 (FZB24-R, Abitep GmbH, Germany) and FZB42 (Abitep GmbH) were used for the field trials due to their high bacterial concentration (10^9-10^{10} CFU g^{-1}) in commercialized formulation. The high bacterial concentrations of *B. velezensis* in these ready-to-use products help save time and labor in skipping the scale-up steps of PGPR cultivation in bio-reactor and easily apply them under the field conditions.

Table 3: List of rhizobacterial strains used for preliminary screening of PGPR candidates which are able to promote plant growth under greenhouse conditions. The PGPR strains in "commercial products" will be used for the tests under gnotobiotic, greenhouse, towards field conditions, while "lab strains" will be used for gnotobiotic and greenhouse tests only

Name			**PGPR strains**	**Mode of actions**	**References**
Strains in commercial products	1	TwinN	*Azospirillum brasilense* NAB317, *Azorhizobium caulinodans* NAB38, *Azoarcus indigens* NAB04 (Product name: TwinN)	- N_2 fixation, auxin production - Phosphorous and micronutrient solubilization - Soil pathogens suppression - Application for wheat	Mabiotec, UK
	2	BveIT45	*Bacillus velezensis IT45* (Product name: Rhizocell GC)	- Auxin production, phosphorous solubilization - Pathogen resistance improvement - Application for wheat	Ithec Lallemand, France
	3	BveFZB24	*Bacillus velezensis FZB24* (Product name: FZB24-R)	- IAA production, phosphorous solubilization - Pathogen resistance improvement (fungal and bacterial) by inducing various pathogenesis-related (PR) proteins, antibiotic production - Application for wheat	Abitep, GmbH, Germany (Kilian et al. 2000; Idris et al. 2004, 2007)
	4	BveFZB42	*Bacillus velezensis FZB42* (Product name: Rhizo Vital 42)		
	5	Abr	*Azospirillum brasilense* (Product name: Azospirillum)	- N_2 fixation, auxin production and root growth promotion - Application for wheat	SAFS Sustainable organic, India
	6	Ach	*Azotobacter chroococcum* (Product name: Azotobacter)		

Name			PGPR strains	Mode of actions	References
Laboratory strains	7	AbrSp245	*Azospirillum brasilense* Sp245	- IAA production, improvement of root growth and wheat yield - Nutrient uptake improvement - Application for both spring and winter wheat	Dobbelaere et al. 2001
	8	AbrSpBr14	*Azospirillum brasilense* SpBr14	- IAA production, improvement of root growth and nutrient uptake - Application for both spring and winter wheat	Warembourg et al. 1987
	9	Abr65B	*Azospirillum brasilense* 65B	- N_2 fixation - Application for both spring and winter wheat	Swędrzyńska et al. 2000
	10	AchAV	*Azotobacter chroococcum* AV	- IAA production, phosphorous solubilization - Siderophore production - Application for spring barley and oat	Stajković-srbinović et al. 2014
	11	BmeSNji	*Bacillus megaterium* SNji	- IAA production, phosphorous solubilization - Siderophore production - Application for spring barley and oat	
	12	SmeL4	*Sinorhizobium meliloti* L4	- IAA production, phosphorous solubilization - Siderophore production - Application for spring barley and oat	
	13	BveGB03	*Bacillus velezensis* GB03	- IAA production, phosphorous solubilization - Salt stress tolerance improvement - Pathogen resistance improvement by VOCS - Application for winter wheat	Zhang et al. 2014; Ryu et al., 2004

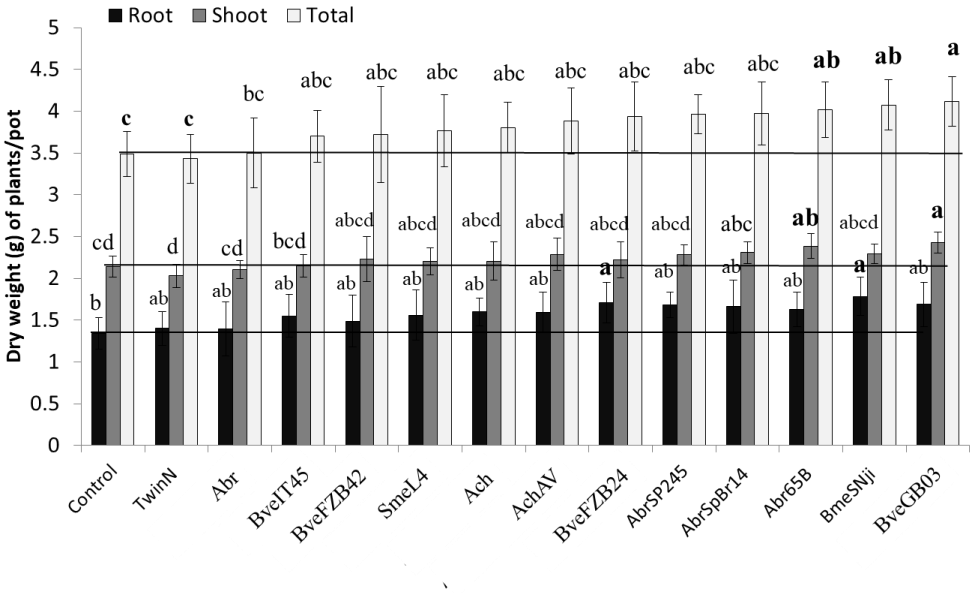

Figure 5: Preliminary screening in the greenhouse to select the PGPR strains for further experiments. 13 PGPR candidates were selected and screened for their growth promotion capacity, which were later ranked according to plant biomass enhancement from low to high: (1) **TwinN** (consortia of *Azospirillum brasilense* NAB317, *Azorhizobium caulinodans* NAB38, *Azoarcus indigens* NAB04), (2) **Abr** (*Azospirillum brasilense*), (3) **BveIT45** (*Bacillus velezensis* IT45), (4) **BveFZB42** (*Bacillus velezensis* FZB42), (5) **SmeL4** (*Sinorhizobium meliloti* L4), (6) **Ach** (*Azotobacter chroococcum*), (7) **AchAV** (*Azotobacter chroococcum* AV), (8) **BveFZB24** (*Bacillus velezensis* FZB24), (9) **AbrSp245** (*Azospirillum brasilense* Sp245), (10) **AbrSpBr14** (*Azospirillum brasilense* SpBr14), (11) **Abr65B** (*Azospirillum brasilense* 65B), (12) **BmeSNji** (*Bacillus megaterium* SNji), and (13) **BveGB03** (*Bacillus velezensis* GB03). The experiment was performed in May 2014. 13 PGPR strains were individually inoculated to spring wheat seeds sown in soil mixture (3 soil:1 sand, w/w) without additional fertilizer. The plants were harvested after 30d (Zadoks 30–32). One-way ANOVA test was followed by Tukey Comparison Test (α=0.05). Groups that do not share a letter have a mean difference that is statistically significant. The values are the means of 8–10 pots for PGPR treatments and 14–15 pots for controls ± SD.

3.2 Wheat

Wheat (*Triticum aestivum* L.) is one of the major cereal crops in the world, with over 600 million tons being harvested annually, besides rice and maize (International Grain Council, 2017-2018). Wheat is grown according to the degrees of latitudes of 30–60°N and 27–40°S. It includes spring and winter wheat varieties which differ from each other in terms of vernalization. The winter wheat varieties require to be exposed to a long cold period for vernalization (<5 °C, during the winter) to be able to get flowering, while the spring wheat varieties do not need this vernalization period and they could be grown from spring time. Different wheat varieties, particularly with winter wheat cultivars, are commonly grown in Europe due to its temperate climate adaptation.

Therefore, we choose wheat as a target crop in Europe to develop and implement PGPR in order to improve the crop yield and nutrient uptake. Winter wheat is commonly grown in Belgium and it will therefore be used for field trials, while spring wheat is used for experiments in greenhouse because it does not need the vernalization to accelerate flowering.

The determination of wheat developmental stages is critical for application of fertilizer, plant growth regulators, and pesticides. The successive developmental stages of wheat are internationally described in Zadoks growth scale from 0-99 (Zadoks et al. 1974) (Figure 6). This scale is used for farm advisory purposes, particularly to times of the application of fertilisers, agro-chemicals as well as biostimulants.

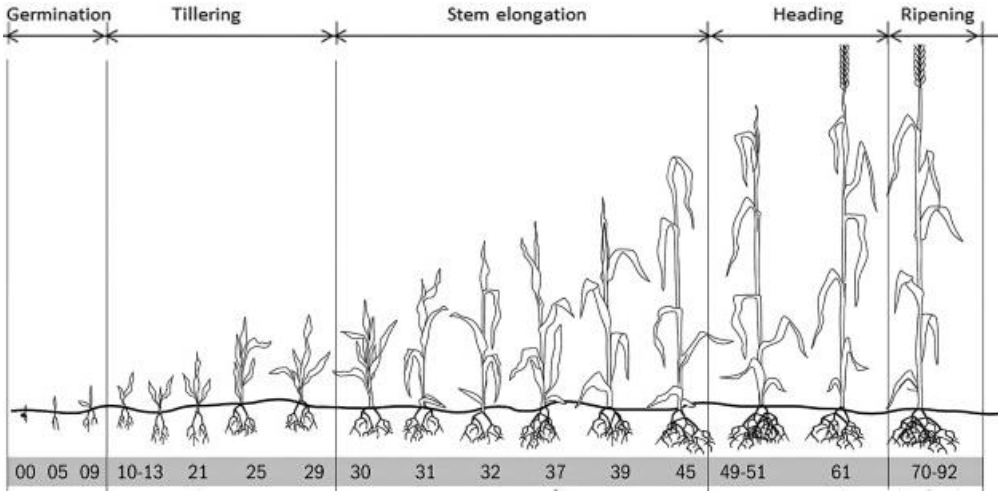

Figure 6: Zadoks' growth stages of wheat as proposed by Zadoks et al. (1974).

3.3 Mineral N fertlizer

Nitrogen is the most limiting nutrient in soil, followed by phosphorus (Schachman et al., 1998). The optimum plant growth and productivity cannot be achieved without N supply. The most easily absorbed forms of N for plants are inorganic nitrogen compounds, i.e. NH_4^+, NO_2^-, and NO_3^-, but these inorganic nitrogen compounds represent less than 5% of the total N in soil (Brady et al., 2008). Besides, organic N fertilizers (e.g. manure compost) are also applied to soils. Afterwards, the mineralization begins and finally release in forms of inorganic nitrogen.

Plants take up nitrate (NO_3^-) from the rhizosphere through three successive steps (Trčková et al., 2006; Gojon et al., 2011). Firstly, nitrate is actively transported across plasma membrane into root cells by several specific membrane nitrate transporters (NRT). The intracellular NO_3^- can either be stored in the vacuoles or metabolized by reduction firtly to nitrite (NO_2^-) and then to ammonium (NH_4^+) by nitrate and nitrite reductases (NR and NiR). It is then transported to leaves for next metabolic use. In roots of cereal, the NO_3^- reduction is quite low, but most of the nitrate taken up from roots is generally translocated via xylem to the leaves to be assimilated into amino acids, proteins or other metabolites or stored in vacuole.

N uptake and accumulation should be considered in terms of the sink-source concept. All organs producing photosynthesis (i.e., leaf blades, leaf sheathes, internodes) at vegetative stages are main sink organs for N taken up by source roots, while after anthesis such photosynthesis-producing organs become source organs and the developing kernels are the main sink organ for N allocation during grain filling (Martre et al., 2003; Tegeder et al., 2018). This determination in N allocation is important to estimate the N use/uptake efficiency by wheat (see the definitions in chapter 1, section 1.1.3-ii: Impact of PGPR on nutrient enrichment and nutrient uptake efficiency).

Nitrate is transported in plant cells by nitrate transceptors (transporter/receptor) including NRT1 and NRT2 families (Gojon et al., 2011; Tegeder et al., 2018). Nitrate transceptors contribute to nitrate uptake, regulate the expression levels of several nitrate assimilation pathway genes, modulate root system morphology by regulating the auxin transport in root cells according to nitrate levels in the soil solution, and protect plants from ammonium toxicity (Bouguyon et al., 2015). Krouk et al. (2010) suggested a model of signalling role of NRT1 in the NO_3^- regulation of lateral root development which depends on the level of external NO_3^- concentration. At low NO_3^- availability (<0.2 mM), NRT1.1 prevents auxin accumulation in young

lateral roots, which, in turn, represses the growth of lateral roots. However, increasing NO_3^- concentration (e.g., >1.0 mM) gradually reduces auxin transport by NRT1.1, therefore increasing the auxin accumulation in the lateral roots and triggering stimulation of lateral root growth. Two groups of nitrate transceptors genes –*TaNRT1* and *TaNRT2* – were identified in wheat (Guo et al., 2014). Group *TaNRT2* are high affinity transport systems, while group *TaNRT1* is not specific and generally low affinity systems.

In wheat, nitrate uptake rate increases from germination to the end of tillering and reaches maximum just before anthesis, and subsequently decreases. More than 80% of the total N content accumulates at anthesis for grain filling (Gebbing and Schnyder, 1999). Split application of mineral N fertilizers is recommended in order to maximize N fertilizer uptake by wheat through the developmental stages (Boman et al., 1995) (Figure 7).

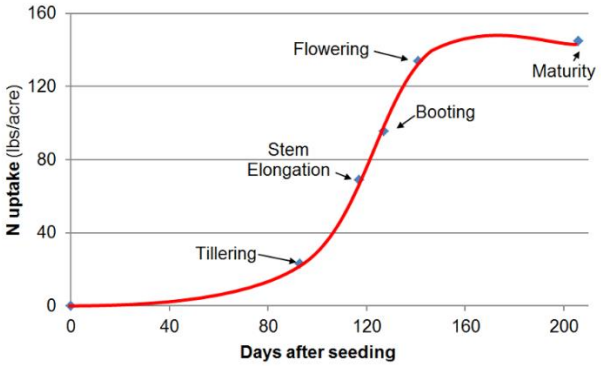

Figure 7: The N uptake of wheat was determined by harvesting and analyzing the N content in the aboveground biomass of wheat plants at different developmental stages from sowing to maturity. The wheat plants take up approximately 25% of N supply until tillering, while most N supply (up to 75%) is taken up from tillering to flowering stages (McGuire et al., 1998).

However, less than half of the applied N fertilizer in the farms is effectively absorbed by crops, and 60-90% of inorganic N fertilizers are generally lost by running off, leaching, erosion, and volatilization (Adesemoye & Kloepper 2009). Consequently, PGPR-based products have been developed to enhance the root growth and N nutrient efficiency as well as reduce the agro-ecological risk derived from excessively mineral N supply (Le Mire et al., 2016). However, the positive performance of PGPR inoculation is proven to be affected by the rate of mineral N fertilization, particularly at low rate of N fertilization supply (Veresoglou and

Menexes 2010). Therefore, the impact of mineral N fertilization levels on the performance of PGPR as well as the impact of PGPR on the N uptake efficiency are included in this PhD thesis.

3.4 Soil, an appropriate substrate for PGPR screening

In this PhD thesis, a field-derived soil was favored over other growing substrates, such as commercial composts, perlite, or sand for pot growing in greenhouse experiments. This ensures a realistic rooting medium for wheat plants and growth conditions for PGPR inoculants as close as the ones observed in the soil of the field trials. The use of other potting materials (e.g., commercial composts, perlite, or sand) may lead to unrealistic growth conditions either for the roots, the PGPR or both, which could consequently lead to nonreproducible results from greenhouse torwards field trials due to the use of inconsistent growing substrates in contrasted cultivation systems. Therefore, the soil samples for pot growing in greenhouse were collected directly in the field trials (Bordia, Gembloux, Belgium) with an expectation to obtain reproducible results from greenhouse torwards field trials.

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4

Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake depend on N application and plant development

4 Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake depend on N application and plant development

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Abstract

The capacity of plant growth-promoting rhizobacteria (PGPR) – *Bacillus velezensis* GB03 (BveGB03), *B. megaterium* SNji (BmeSNji), and *Azospirillum brasilense* 65B (Abr65B) – to enhance growth and nutrient uptake in wheat was evaluated under different mineral N fertilizer rates, in sterile and non-sterile soils, and at different developmental stages. In gnotobiotic conditions, the three strains significantly increased plant biomass irrespective of the N rates. Under greenhouse conditions using non-sterile soil, growth promotion was generally highest at a moderate N rate, followed by a full N dose, while no significant effect was observed for the inoculants in the absence of N fertilizer. At 50N, plant biomass was most significantly increased in roots (up to +45% with Abr65B) at stem-elongation stage and in the ears (+19-23% according to the strains) at flowering stages. For some nutrients (N, P, Mn, and Cu), the biomass increases in roots and ears were paralleled with reduced nutrient concentrations in the same organs. Nevertheless, growth stimulation resulted in a higher total nutrient uptake and higher nutrient uptake efficiency. Furthermore, Abr65B and BmeSNji counteracted the reduction of root development caused by a high N supply. Therefore, combining PGPR with a proper cultivated system, N rate, and plant stage could enhance their biostimulant effects.

Keywords: Biostimulant, Plant growth-promoting rhizobacteria (PGPR), Fertilizer use efficiency, Plant nutrient, Nitrate-dependent root inhibition

4.1 Introduction

Mineral fertilizers are critical for maximizing crop production and food quality, and their application rate in agriculture has been steadily increasing in recent years. However, the fertilizer use efficiency of crops, especially in terms of the use of N and P, is often low due to the large proportion (60-90%) of the applied fertilizer generally lost through leaching, erosion, phosphorus precipitation, and volatilization. These processes, in turn, result in serious environmental problems (Adesemoye & Kloepper 2009). In order to enhance nutrient uptake efficiency in crops, plant growth-promoting rhizobacteria (PGPR)–which are one of the main categories of biostimulants (du Jardin 2015)–have been proposed as an effective agro-ecological solution for such environmental issues (Le Mire et al. 2016).

It has been proposed that the increase in root growth as well as total root surface caused by inoculation with PGPR is a key tool for increasing nutrient uptake efficiency (Adesemoye & Kloepper 2009). Such morphological changes in roots are mainly caused by phytohormones or analogs produced by PGPR, such as indole-3-acetic acid (IAA), cytokinins, gibberellins, and reducing ethylene production by ACC deaminationa (Duca et al. 2014), in which IAA plays a central role (Spaepen et al. 2008). Moreover, root morphology also depends on nutrient availability, especially in terms of the macro-nutrients N, P, and K. For example, in maize, N deficiency results in increased root growth, with longer primary, seminal, and nodal roots developing to reach a greater soil volume and exploit more N. On the other hand, excessive N applications inhibit root development (Tian et al. 2008). This inhibitory effect of high N concentrations on lateral root growth has also been found in *Arabidopsis* (Hermans et al. 2011). Interestingly, inoculation with PGPR could restore such high N-dependent suppression in *Arabidopsis* when grown *in vitro* (Mantelin et al. 2006), but the effect remains to be evaluated under real soil conditions.

Most studies reporting on the impact of N fertilization on the plant growth-promoting activity of PGPR on wheat have indicated maximal growth stimulation in the absence of N fertilization, in both greenhouse and field trials (Shaharoona et al. 2008; Veresoglou & Menexes 2010; Hussain et al. 2016; Spolaor et al. 2016). Therefore, we aimed to answer the following questions:

(1) *Does the absence of N fertilizer maximize the effect of PGPR on wheat growth under gnotobiotic and greenhouse conditions?*

(2) *Which developmental stages of wheat are more responsive to the biostimulant effects of PGPR?*

(3) *To what extent does PGPR-driven stimulation of growth result in an increase in nutrient uptake and/or tissue concentrations of nutrients?*

To answer these questions, experiments were designed using three PGPR strains selected from preliminary greenhouse experiments. These were then combined with different N rates and analyzed at different plant stages in different plant organs, under gnotobiotic conditions with sterile soils and under greenhouse conditions with non-sterile soils.

4.2 Materials and methods

4.2.1 Soil preparation and N supply rates

The aim was to use a substrate which ensured a realistic rooting pattern as close as possible to one which would be observed in the field. Field soils were thus favored over commercial composts, perlite, or sand. Soil samples were collected from the top layer (0-30 cm depth) at Bordia, Gembloux, Belgium (50°34'05.8"N, 4°42'33.0"E), an area belonging to the oceanic temperate region. The soil samples were taken from the same plots over two years, in which the previous crop in season 2014 (soil I) was winter wheat (*Triticum aestivum* cv. Forum), and in season 2015 (soil II) had been winter pea (*Pisum sativum* cv. Enduro). The characteristics of these two soils were determined, as described in the "Soil physicochemical and plant nutrient analysis" section below, to contain 22% clay, 69% silt, and 9% sand, with the chemical properties shown in Table 4 . These two soils were used for greenhouse trials, and only soil I was used for Magenta box cultivation. The soil mixture used for all experiments was prepared by mixing three volumes of dried soil with one volume of dried sand in a cement mixer, to partially restore the soil's macroporosity. The sterile soil used for Magenta box cultivation was prepared by depositing thin layers (less than 2 cm) of soil I mixture in trays, covered by aluminum foil, and autoclaved three times at 121 °C for 1 h on alternate days, with overnight incubation in a laminar flow cabinet in between.

Three different N rates – 0%, 50%, and 100% (subsequently referred to as 0N, 50N and 100N) – of the recommended full N dose were used. Recommended full N dose for wheat in our field was ≃185 kg N ha^{-1}, which was equivalent to 92.5 mg N $plant^{-1}$ calculated on the basis of plant density in the field. A half dose of N was mixed with water and applied at sowing (for both plants grown in Magenta boxes and in the greenhouse), and the other half was applied after 30 days at booting stage (for plants grown in the greenhouse). As a result, the plants harvested after 30 d

received only half of the corresponding N dose. The mineral N fertilizer used in all experiments was ammonium nitrate (Nitrate d'Ammoniaque calcaire 27%, containing 27% N with 13.5% nitrate and 13.5% ammonium, Brichart S.A., Sombreffe, Belgium).

Table 4. Mean values of soil chemical properties in the top 30 cm layer. Both soils were used for pot cultivation in the greenhouse, while only soil I was used for Magenta box cultivation.

	Previous crop	Total N (g kg^{-1})	N-NO_3^- (mg kg^{-1})	C organic total (g $100g^{-1}$)	pH KCl	P	K	Mg	Ca	Cu	Zn	Fe	Mn
						mg $100g^{-1}$				mg kg^{-1}			
Soil I	Wheat	1.02	5.9	1.13	7.2	22.0	15.0	11.0	240	2.0	2.0	352	273
Soil II	Pea	1.34	17.7	1.37	6.9	24.0	19.6	9.7	268	3.3	11.3	223	219

4.2.2 PGPR strains

Three PGPR strains – *Bacillus velezensis* GB03 (BveGB03) (formerly referred to as *B. amyloliquefaciens / subtilis* GB03; *Fan et al.*, 2017), *Bacillus megaterium* SNji (BmeSNji), and *Azospirillum brasilense* 65B (Abr65B) – were selected from preliminary screenings in greenhouse trials (Figure 8). Seven strains promoting the growth of wheat, barley and oat in previous studies were evaluated in the preliminary screening. *Sinorhizobium meliloti* L4, *Azotobacter chroococcum* AV, and *Bacillus megaterium* SNji (Stajković-Srbinović et al. 2014) were kindly provided by Dr Stajković-Srbinović (Institute of Soil Science, Belgrade, Serbia). *Azospirillum brasilense* Sp245 (Baldani et al. 1987) and *Azospirillum brasilense* SpBr14 (Warembourg et al. 1987) came from the Katholieke Universiteit Leuven collection (Leuven, Belgium). *Azospirillum brasilense* 65B (Swędrzyńska 2000) was kindly provided by Dr Swędrzyńska (August Cieszkowski Agricultural University of Poznah, Poland). *Bacillus velezensis* GB03 (Zhang et al. 2014) was kindly provided by Dr. Paul W. Paré and Dr. John McInroy (Texas Tech University, Lubbock, TX, USA). Bacteria were grown overnight in tryptone soy broth (TSB, Sigma-Aldrich, St. Louis, Missouri, US) and cells were harvested by centrifugation (1735 ×g, 15 min) and resuspended in a sterile phosphate buffer (8.80 g NaCl, 1.24 g K_2HPO_4, and 0.39 g KH_2PO_4 L^{-1}).

Spring wheat grains were individually inoculated with the PGPR strains and then sown in soil I mixture without additional fertilizer, while the same volume of sterile phosphate buffer without bacteria was used as a control. The plants were grown as described in the "Greenhouse experiments" section below. The plants were harvested after 30 days of growing at Zadoks stages 30-31. Two experimental replicates of the preliminary screening were performed with the first test in May 2014 (Figure 8A) and the second test in October 2014 (Figure 8B). Based on the most significant increases in root, shoot, and total biomass from both experimental replicates, the three best strains were selected. The first preliminary test was performed in May 2014, with an average greenhouse temperature of 23.2 ± 2.5 °C, and the second test took place in October 2014, with an average temperature of 21.9 ± 0.2 °C. As well as the impact of PGPR, the warmer and longer days in May could have enhanced wheat growth, compared to the October experiment. Further experiments in this study were therefore carried out in months with long days [March to May 2015 for test in soil I (Fig. 12) and August to beginning of October 2015 for test in soil II (Fig. 13)].

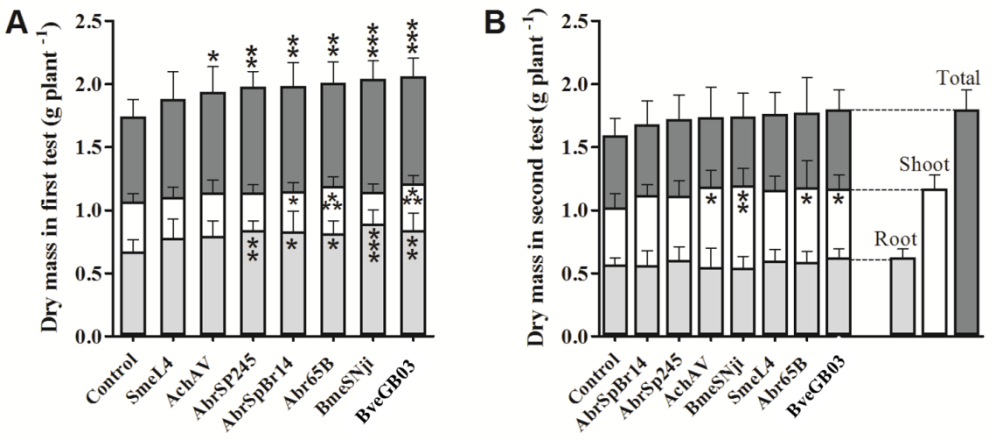


Figure 8. Preliminary screening in the greenhouse to select the three best PGPR strains. Seven PGPR candidates, including *Sinorhizobium meliloti* L4, *Azotobacter chroococcum* AV, *Azospirillum brasilense* SP245, *Azospirillum brasilense* SpBr14, *Azospirillum brasilense* 65B, *Bacillus megaterium* SNji, and *Bacillus velezensis* GB03, were screened for their growth promotion capacity. Two experimental replicates were performed with the first test (A) in May 2014 and the second test (B) in October 2014. Seven PGPR strains were individually inoculated to spring wheat seeds sown in mixture of soil I without additional fertilizer and grown as described in the "Greenhouse experiments" section. The plants were harvested after 30d at Zadoks 30–32. Based on the most significant increases in root, shoot and total dry biomass, the three best strains were selected. Asterisks denote statistical significance between treatments with PGPR to their respective control in root, shoot, and total dry biomasses. One-way ANOVA test was followed by Dunnett's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The values are the means of 8–10 pots for PGPR treatments and 14–15 pots for controls ± SD. The overlapping bars presesenting root, shoot and total biomass have the same base line at 0 (g plant^{-1})

4.2.3 *In vitro* growth in Magenta boxes

Spring wheat grains (*Triticum aestivum* L. cv. Tybalt) were surface sterilized according to a protocol modified from (Gfeller et al. 2013). Briefly, the grains were submerged for 30 s in 70% ethanol and then rinsed five times with sterile water. Seeds were subsequently incubated in $AgNO_3$ (1% w/v) at 200 rpm for 20 min, then rinsed five times with sterile water and stored in sterile water for 30 min at 200 rpm. Afterwards, the seeds were treated with 3% hypochlorite (NaClO) solution for 20 min at 200 rpm, then rinsed five times with sterile water, and left in sterile water for 30 min at 200 rpm. Grains were germinated on moist sterile filter paper in Petri

dishes and placed in the dark at room temperature (20-22 °C) for 36 h. Finally, the grains were dipped in 10^8 CFU (colony-forming unit) mL^{-1} bacterial suspension in a phosphate buffer for 30 min and five grains were transferred to Magenta boxes (size GA-7; 75 mm × 75 mm × 97 mm, Sigma Chemical Co., St. Louis, MO). A plastic collar (75 mm × 75 mm × 20 mm) was used to couple the two chamber halves together, and the resulting boxes were filled with 100 g of autoclaved soil. Different N rates were added, in which the full N dose was equivalent to 92.5 mg N per kg of soil, as calculated according to the "Greenhouse experiments" section. The sterile phosphate buffer without bacteria was used as a control. The boxes were placed in a growth chamber with a mean temperature of 23 °C for a 16 h photoperiod with an average PPFD of ≃150 µmol $m^{-2}s^{-1}$. The plants were harvested after two weeks and the fresh biomass was measured immediately after each plant was removed from the Magenta box, in order to avoid water evaporation.

The rhizosphere PGPR colonization was assessed with a modified protocol from (Chowdhury et al. 2013, p. 42). Loosely adhering soil was removed by hand-shaking from the roots, with the remaining soil surrounding the roots considered to be rhizosphere soil. Three grams of fresh roots and rhizosphere soil were suspended in 10 ml sterile phosphate-buffered saline (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, water 1000 ml, pH 7.3) in 100 ml sterile Erlenmeyer flasks containing 15 glass beads (0.6 mm in diameter) and shaken vigorously on a rotary shaker for 1h at 300 rpm. The suspension was plated afterwards in serial 10-fold dilutions on TSA medium. Plates were incubated at 28 °C for 24-48 h and total colonies were then counted and expressed as CFU per g of fresh root and rhizosphere soil.

4.2.4 Greenhouse experiments

All pot cultivations were conducted in the greenhouse at the Plant Biology laboratory of Gembloux Agro-Bio Tech. Grains of spring wheat (*Triticum aestivum* cv. Tybalt) were germinated on moist filter papers in petri dishes for two days. Five grains were then sown in a pot made with a cut PVC tube (30 × 9 cm) filled with 2 kg of soil mixture in a plastic bag, and then thinned into two homogenous plants per pot, four days after sowing. One mL of 1×10^8 CFU mL^{-1} bacterial suspension was added to each four-day-old plant and re-inoculated every two weeks with the same concentration. The sterile phosphate buffer without bacteria was used as a control. The pots were watered daily and maintained at 13-15% (w:w) humidity in soil, with an air humidity of 40-60%. A 16 h photoperiod was used with an average PPFD of ≃170 µmol $m^{-2}s^{-1}$ measured at the canopy level (daylight in greenhouse

supplemented with LED light, Vegeled Flood Light BX151, 150 W, with passive cooling 100-300 VAC, Colasse SA, Seraing, Belgium). The mean greenhouse temperature was 23.4 ± 4.9 °C for the first 30 d and 22.3 ± 5.5 °C for last 30 d (from March to May 2015) in the experiment with soil I (Fig. 12), and 24.7 ± 4.0 °C followed by 22.6 ± 3.1 °C (from August to beginning of October 2015) in the experiment with soil II (Fig. 13). The overall experiments were designed in completely randomized blocks with eight replicates per treatment. Dry weight of plants was measured after drying at 60°C until it remained constant.

4.2.5 Soil physicochemical and plant nutrient analysis

Both soil types were analyzed as follows. Soils were air-dried, then sieved at 2 mm and crushed to 200 µm. Particle size of clay, silt, and sand fractions was measured by the pipette method (AFNOR NF X31-107). Soil pH was determined with 1N KCl (ISO 10390-2005). Total organic carbon was measured according to the Walkley and Black method (modified ISO 14235-1998) and total N according to the Khjeldahl method (ISO 11261-1995). Soil NO_3^- was measured by the QuickChem® method (12-107-04-1-B). Available major (P, K, Mg, Ca) and trace (Fe, Cu, Zn, Mn) elements were measured after extraction with CH_3COONH_4 (0.5 M) and EDTA (0.02 M) at pH 4.65 (w:v 1:5 ratio) and agitation for 30 min (referred to as available metal concentration) (Lakanen & Erviö 1971). Elemental concentrations were quantified by colorimetry (at 430 nm) for P, and flame atomic absorption spectrometry (VARIAN 220, Agilent Technologies, Santa Clara, CA, USA) for K, Mg, Ca, Fe, Cu, Mn, and Zn.

Regarding plant nutrient content analysis, plant samples were ground into powder, with 2 to 5 g of the powder digested in a mixture of concentrated HNO_3 and $HClO_4$ (15 ml of mixture for each g of plant powder). Afterwards, the element concentrations were measured by colorimetry (P) and flame atomic absorption spectrometry (other elements). All element concentrations were calculated based on the dry weight of soil or plant samples. N uptake efficiency was calculated as total N content (mg) in the plant per the sum of applied N (50% N) and total N available in soil (Hirel et al. 2001). The values were the mean of three replicates from a pool of eight pots, with 16 plants per treatment. The root samples after 60 d were not used for this analysis due to the aging of roots at mature stages (Zadoks 65-70) preventing adequate sampling.

4.2.6 Measurements of Indole-3-acetic acid (IAA) production and phosphate solubilization

IAA production capacity of PGPR was determined as in (Sarwar et al. 1992). For this purpose, 15 ml falcon tubes containing 11 ml of autoclaved TSB (T8907, Sigma-Aldrich) were inoculated with 0.5 ml of one-day-old bacterial suspension, adjusted to 10^8 CFU ml^{-1} and supplemented with or without tryptophan (1g L^{-1}). The tubes were incubated in triplicate at 30 °C for 24 h, 48 h, and 72 h at 150 rpm. TSB was only kept for comparison. After incubation, bacterial suspensions were sampled for cell density measurement by spectrophotometry at 540 nm to normalize the IAA concentrations. The cells were removed by centrifugation at 1735×g for 15 min. The IAA concentration was measured by mixing 0.6 ml of supernatant with 0.4 ml Salkowski reagent, which was then incubated for 30 min at 25 ^oC in the dark. Absorbance was measured at 530 nm with a microplate spectrophotometer (Spark 10M**,** Tecan Trading AG, Männedorf, Switzerland). Concentrations of IAA produced by PGPR were estimated by using a standard curve of IAA (I2886, Sigma-Aldrich) in the range of 10-100 µg mL^{-1}. The experiment was performed with six replicates.

Phosphorus solubilization by PGPR was determined in the National Botanical Research Institute's phosphate growth medium (NBRIP), containing 0.5% insoluble phosphate $Ca_3(PO_4)_2$. Bacteria were added to NBRIP with a final cell density at 10^7 CFU ml^{-1}. Solubilized P and pH were measured after 4, 5, and 6 days of incubation at 30 °C in a shaking incubator. Soluble phosphate was determined using the method of (Murphy & Riley 1962).

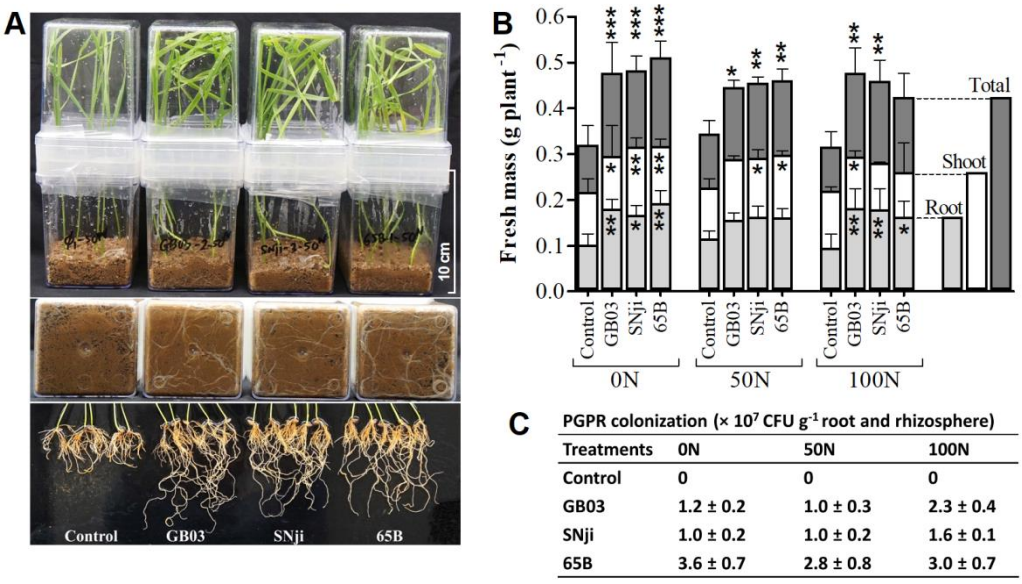
4.2.7 Statistical analysis

The data for root, shoot, ear, and total biomass of plants supplied with different N rates in Magenta box and pot experiments were analyzed using a two-way ANOVA with two factors– inoculations (3 PGPR and control) × N rates (0, 50, 100N)– followed by Bonferroni test. The block factor was excluded due to the absence of interaction in blocks × inoculations (p values were > 0.90) or in blocks × N rates (p values were 0.35-0.9) in all experiments. The data for nutrient content and N uptake efficiency were analyzed using a one-way ANOVA test followed by Dunnett's multiple comparison test. These analyses were performed using GraphPad Prism 7.03 (GraphPad Software Inc., Suite 230 La Jolla, CA, USA).

4.3 Results

4.3.1 Effects of PGPR on wheat supplied with different N rates under gnotobiotic conditions

Surface-sterilized wheat grains were individually inoculated with the PGPR strains and that was generally able to increase fresh biomass of plants grown on sterilized soil in Magenta boxes for two weeks (Figure 9A, B). Specifically, the three strains significantly increased the biomass of all plant parts (root, shoot, and total) at 0N. At 50N, the three strains also significantly increased the total biomass. The same held true for 100N, in which the three strains significantly increased root biomass. Noticeably, these positive effects on biomass production were similar among the three strains, compared to the non-inoculated control, and also similar between 0, 50, and 100N. After two weeks, the three PGPR strains were able to colonize the roots and rhizosphere with density of $1.0–3.6\times10^{7}$ CFU g^{-1} of fresh root and rhizosphere soil (Figure 9C).



C

PGPR colonization (× 10^7 CFU g^{-1} root and rhizosphere)

Treatments	0N	50N	100N
Control	0	0	0
GB03	1.2 ± 0.2	1.0 ± 0.3	2.3 ± 0.4
SNji	1.0 ± 0.2	1.0 ± 0.2	1.6 ± 0.1
65B	3.6 ± 0.7	2.8 ± 0.8	3.0 ± 0.7

Figure 9. Effects of *Bacillus velezensis* GB03 (BveGB03), *B. megaterium* SNji (BmeSNji), and *Azospirillum brasilense* 65B (Abr65B) inoculations on the early development of plants grown in soil I under gnotobiotic conditions.Sterilized grains were inoculated with PGPR and supplied with 0, 50, and 100N fertilizer. (A) Representative images of 14-day-old plants with their corresponding root systems, seen through the bottom of Magenta boxes, and the washed roots at 50N. The results were similar across 0, 50, and 100N. (B) Root, shoot, and total fresh biomass after two weeks. (C) Colonization of PGPR in root and rhizosphere soil after two weeks. The values are the means of three pots ± SD. The experiments were repeated twice with similar results (only one replicate is presented). Asterisks denote statistical significance between PGPR treatments, compared to their respective controls at each N rate. Two-way ANOVA was performed for root, shoot, and total biomass, and followed by Bonferroni test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). In (B), the overlapping bars presenting root, shoot, and total biomasses have the same baseline at 0 g plant^{-1}.

4.3.2 Effects of PGPR on wheat supplied with different N rates under greenhouse conditions

Growth of the non-inoculated control plants displayed a marked response towards different N rates at both young (30 d, Zadoks 30-32, Figure 12A and 13B) and flowering stages (60 d, Zadoks 65-70, Figure 12C, D and 131D, E). Generally, there was a significant increase in the biomass of control plants supplied with 50 and 100N rates at both 30 d and 60 d, except for the roots of the control in soil II after 30 d, which was significantly decreased when 100N was applied (Figure 12A, C and 13A, B, D). Likewise, higher N supply resulted in higher numbers of tillers and ears, but that consequently reduced the biomass per tiller at the young stage (30 d). Noticeably, this reduction in tiller mass was more pronounced in soil II than in soil I (Figure 10 and Figure 11).

At young stages (30 d, early stem elongation, Zadoks 30-32), the increase in dry biomass of inoculated plants compared to the non-inoculated control was most pronounced at 50N, followed by 100N, and lowest at 0N in both soil types (Figure 12 and 13). Such effects were more pronounced in roots than in shoots. Particularly, in soil I, Abr65B and BmeSNji coupled with 50N significantly increased root biomass by 45% and 28%, respectively, and shoot biomass by 15% and 14% at 50N, respectively, with the resulting biomass similar to those obtained with the 100N treatment in the absence of inoculant (Figure 12A). The same holds true for Abr65B, with a 27% increase in root biomass at 100N. At 0N, three strains also increased root biomass by 25-26%, but these changes were not statistically significant ($\alpha = 0.05$). Likewise, in soil II, Abr65B and BmeSNji significantly increased root biomass by 25% and 28% at 50N, and by 23% and 23% at 100N, respectively (Figure 13B). After 30 days, BmeSNji significantly increased root-to-shoot ratio at 0N in soil I and at 50 and 100N in soil II, while Abr65B significantly increased it at 50 and 100N in soil I (Figure 12B and 13C). However, there was no impact of PGPR on tiller number and biomass per tiller compared to the corresponding non-inoculated control at each N rate (Figure 10).

Noticeably, in soil II – in which total N and nitrate concentrations were higher than those in Soil I (Table 4) – adding 100N fertilizer significantly reduced root biomass in the non-inoculated control (by -11% at 50N and -18% at 100N compared to that at 0N) after 30 days. However, the inoculations with Abr65B and BmeSNji were still able to significantly increase the root biomass at these high N rates (Figure 13A, B).

At flowering stages (60 d, Zadoks 65-70), the most pronounced biomass increases were also obtained at 50N and 100N, while the lowest changes were

recorded at 0N in both soil types. Particularly in soil I, BveGB03, BmeSNji, and Abr65B significantly increased ear mass by 19%, 23%, and 18% at 50N, respectively; BveGB03 significantly increased ear mass by 16% at 100N (Figure 12D). In soil II, Abr65B increased ear biomass by 14% at 50N and 17% at 100N (Fig. 13E). Moreover, Abr65B significantly increased root mass by 59% at 50N and 23% at 100N (Figure 13D). However, there was no impact of PGPR on ear number when compared to the non-inoculated control at each N rate (Fig. 11). Noticeably, neutral and negative effects on root mass for inoculated plants were observed at 0N in soil I and II, respectively (Figure 12C and 13D)**.** Ear-to-(root and shoot) ratios at 0N, however, were significantly higher with BmeSNji and Abr65B treatments (Figure 13E).

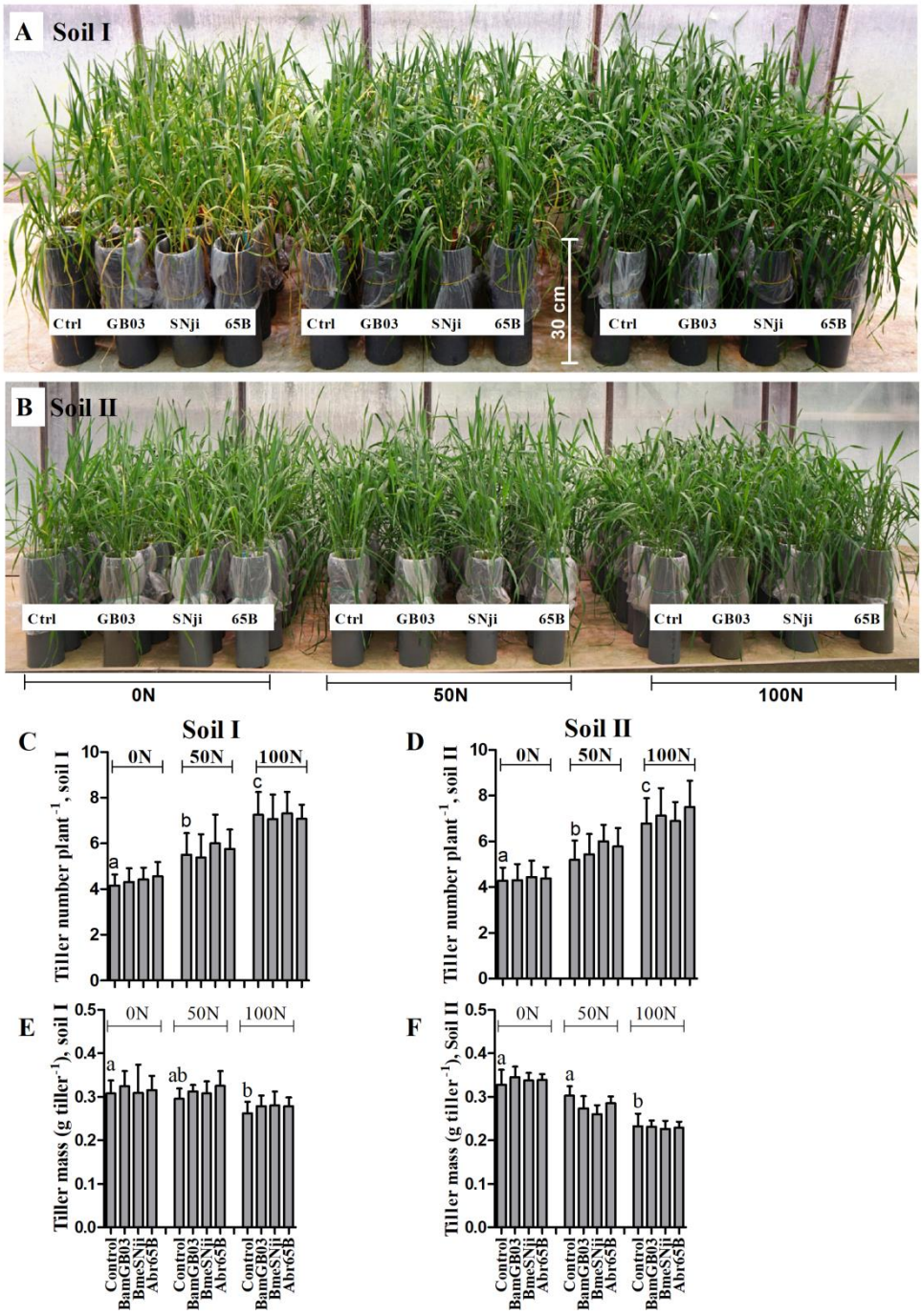


Figure 10– Response of plants inoculated with… (continued on the following page)

Figure 10. Response of plants inoculated with *Bacillus velezensis* GB03, *B. megaterium* SNji, and *Azospirillum brasilense* 65B to different N rates (0, 50, and 100N) 30 days after sowing. The control was treated with sterile phosphate buffer. Representative images of plant response to the supply of different N rates: (A) in soil I in Fig. 12 and (B) in soil II in Fig. 13. (C, D) Tiller number in soil I from A and soil II from B, respectively. (E, F) Tiller biomass in soil I from A and soil II from B, respectively. The values are the means of eight pots ± SD. Means that do not share a letter are significantly different in the control plants supplied with 0, 50, and 100N. In each N group, there was no significant impact of PGPRs on tiller number and tiller biomass compared with their respective non-inoculated control

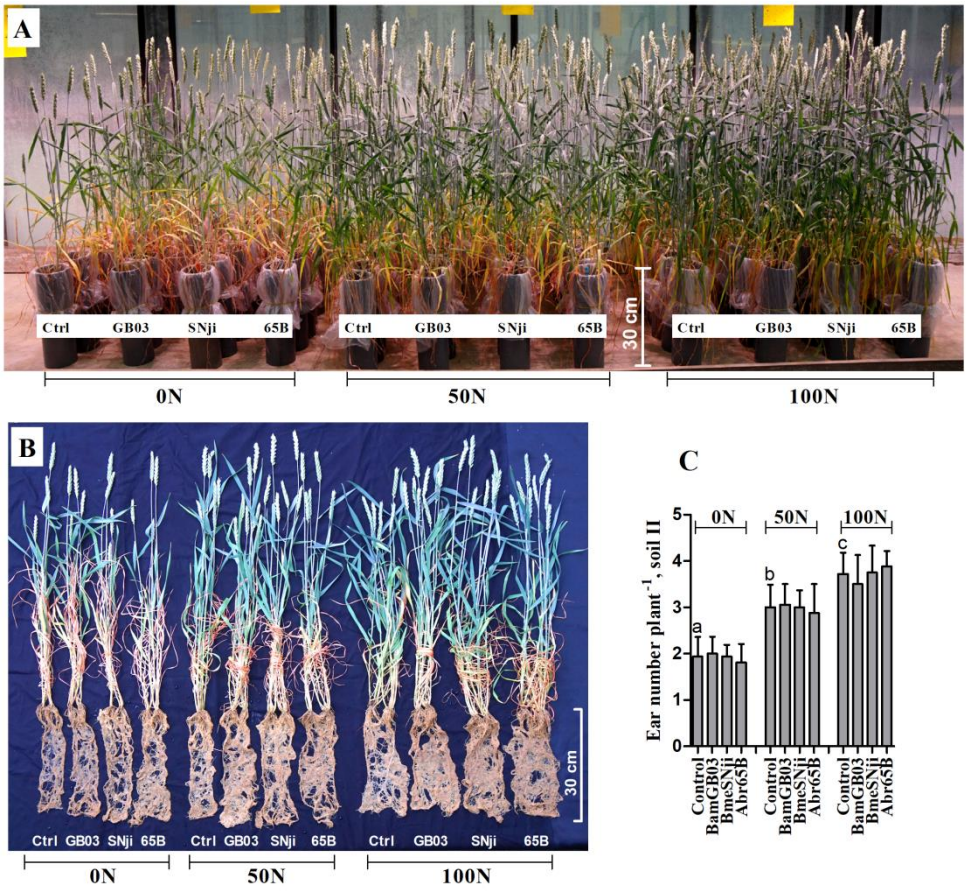


Figure 11. Response of plants inoculated with or without *Bacillus velezensis* GB03, *B. megaterium* SNji, and *Azospirillum brasilense* 65B under different N rates (0, 50, and 100 N) 60 days after sowing (Zadoks 65-70). The response of plants with different N rates at this stage were similar between soil I and soil II, so only images and ear number per plant from soil II were shown. (A) Plants grown in pots until flowering, (B) representative plants of each treatment after washing of roots, and (C) ear number per plant at 60d. The values are the means of eight pots ± standard deviations. Means that do not share a letter are significantly different in the control plants supplied with 0, 50, and 100N. In each N group, there was no significant impact of PGPR on ear number compared with the respective non-inoculated control

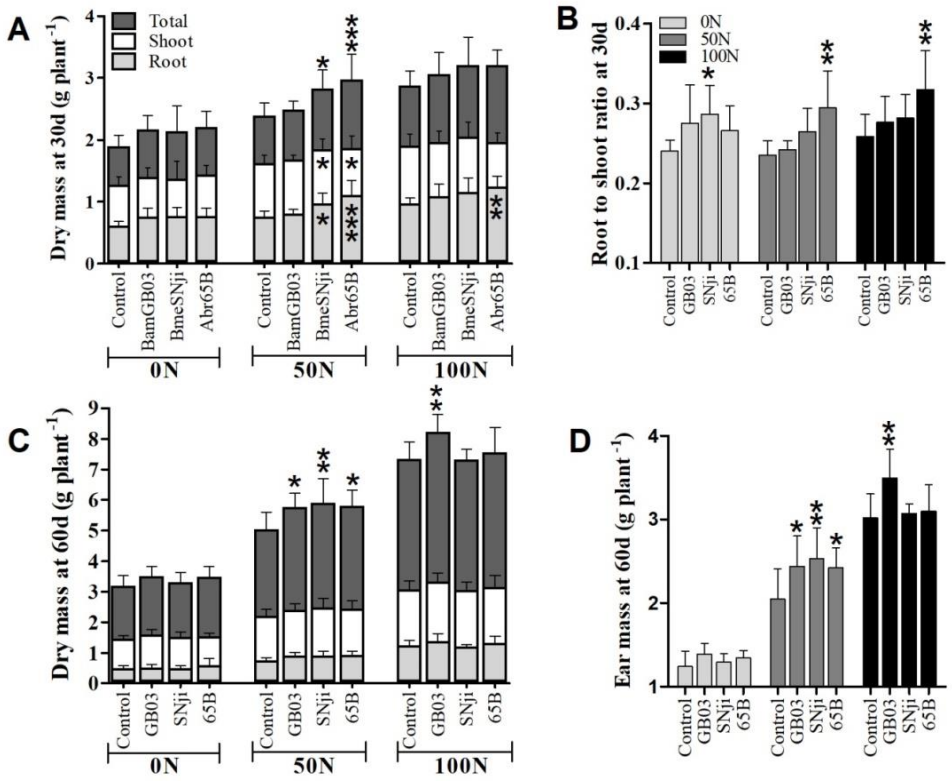


Figure 12. Effects of *B. velezensis* GB03, *B. megaterium* SNji, and *A. brasilense* 65B inoculations on wheat grown in soil I and fertilized with three different N rates (0, 50, and 100N) under greenhouse conditions. (A) Dry biomass at early stem elongation (30 days after sowing, Zadoks 30-32) and (B) the corresponding root-to-shoot ratio. (C) Dry biomass at flowering stage (60 days after sowing, Zadoks 65-70) with root, shoot (including stems and leaves without ears), and total biomasses; and (D) their ear biomass. The values are the means of eight pots ± SD. Asterisks denote statistical significance between PGPR inoculation to their respective control at each N level. Two-way ANOVA was performed for root, shoot, ear, and total biomasses respectively, and followed by Bonferroni test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The overlapping bars (in A, C) presenting root, shoot, and total biomasses have the same baseline at 0 g plant-1

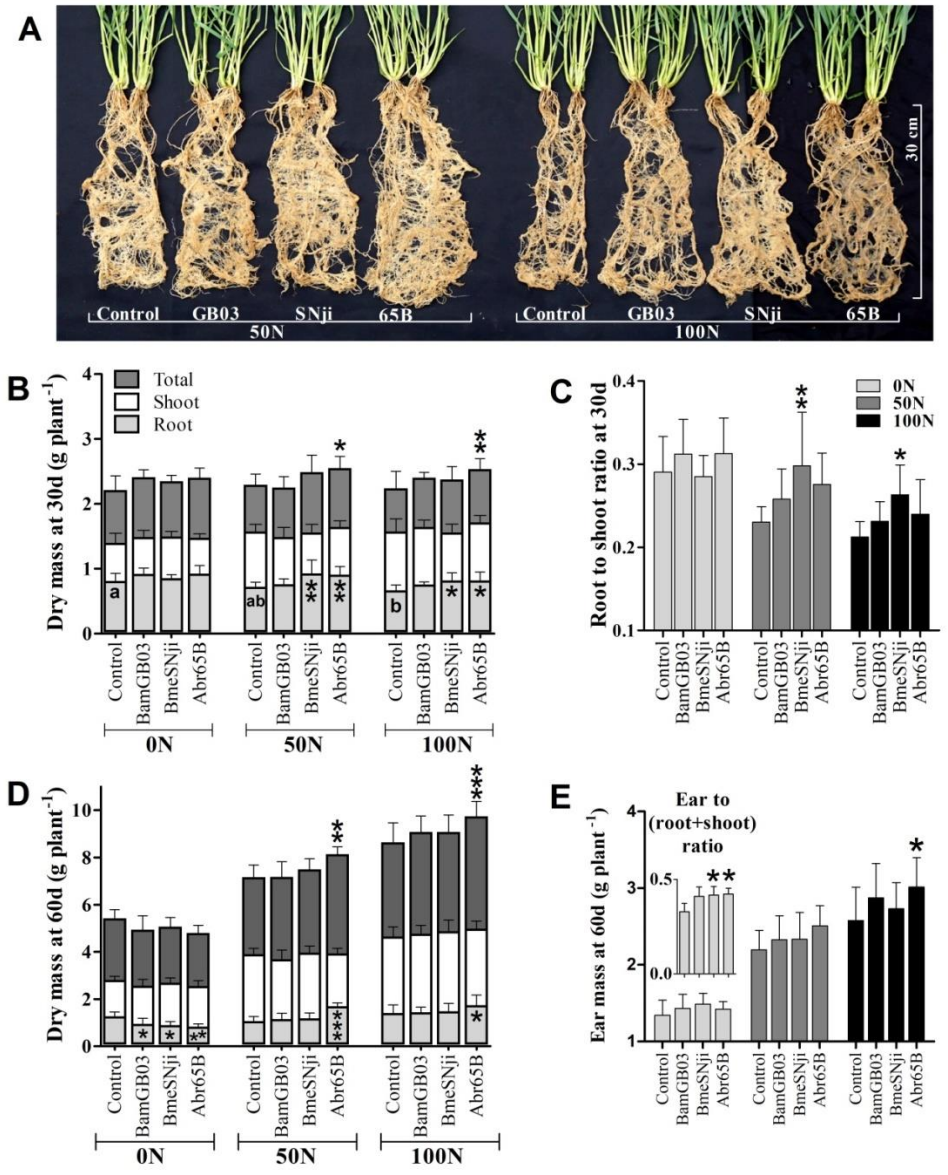


Figure 13. Effects of *B. velezensis* GB03, *B. megaterium* SNji, and *A. brasilense* 65B inoculations on wheat grown in soil II and fertilized with three different N rates (0, 50, and 100N). (A) Representative images of roots fertilized with 50 and 100N at 30 d with and without inoculation. (B) Dry biomass at early development (30 days after sowing, Zadoks 30-32). (C) The corresponding root-to-shoot ratio. (D) Dry biomass at flowering stages (60 days after sowing, Zadoks 65-70), and (E) their ear biomass with a sub-graph of ear-to-(root

and shoot) ratio at 0N. The values are the means of eight pots ± SD. Asterisks denote significance between treatments of PGPR to their respective control at each N rate, while different letters (a, b, c) denote significant differences between three N rates in the control. Two-way ANOVA was performed for root, shoot, ear, and total biomass respectively, and followed by Bonferroni test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The overlapping bars (in B, D) presenting root, shoot, and total biomass had the same baseline at 0 g plant-1.

4.3.3 Effects of PGPR on nutrient uptake

Nutrient uptake was expressed in terms of (1) *nutrient concentration* (g or mg per kg) and (2) *total nutrient content* (mg or µg for whole plant or organ). The aim was to test whether PGPR could increase both biomass and nutrient uptake in the same plant. Therefore, the dry biomass obtained with 50N fertilization in soil I (Figure 12) was selected because, at this rate, PGPR noticably increased biomass at both young and flowering stages, when compared to those at 0N or 100N.

In general, the *nutrient concentration* was unchanged or even decreased in specific organs of inoculated plants (Figure 14A), likely due to the large increase in biomass caused by PGPR treatments, which in turn might dilute some of the elements in the tissues. Specifically, although Abr65B and BmeSNji significantly increased 30-day root and shoot biomasses, they all resulted in non-significant changes or even significantly lowered concentrations in some elements, except for Zn in roots inoculated with BveGB03. Significantly lower concentrations of N, P, and Cu were recorded in plants inoculated with Abr65B, as well as lower concentrations of P and Cu in 30-day roots when inoculated with BmeSNji. Similarly, 60-day ears contained a significantly lower concentration of N in plants inoculated with Abr65B. The same held true for N, P, and Mn with BmeSNji, and P and Mn with BveGB03.

In terms of *total nutrient content*, however, most of the total element contents– defined as the total amounts of elements per plant or per organ (root, shoot, or ear)– were significantly increased due to the increase in biomass promoted by PGPR treatments (Figure 14B). In 30-day roots, in particular, there was a significantly higher content of P, Mg, Fe, Mn, Zn, Cu (when inoculated with Abr65B); P, Mg, Fe, Mn, Zn (with BmeSNji); and Zn, Cu (with BveGB03). Similarly, the 60-day ears contained a significantly higher amount of N, P, K, Mn, Zn (inoculated with Abr65B); N, K, Zn (with BmeSNji); and N, K, Fe, Zn (with BveGB03). Only a few significant increases were found in other organs, i.e., 30-day shoots (N, P with BmeSNji), and in 60-day ears (Zn with BveGB03).

Noticeably, the increase in total micro-nutrient content (i.e., Fe, Mn, and Zn) in inoculated plants was more pronounced than that of macro-nutrients, particularly in 30-day roots (Figure 14B). Specifically, Abr65B and BmeSNji significantly increased the content in Fe (59%, 39% compared to non-inoculated control, respectively), Mn (70%, 37%), Mg (45%, 33%), and Zn (39%, 33%).

Nutrient uptake efficiency (UE) was defined as the proportion of the total nutrient in the external medium (i.e., from the fertilizer and in the growing substrate) being taken up by the plant and contained in its tissues. In general, PGPR which significantly increase plant biomass also increase the nutrient uptake efficiency at both young and flowering stages. Specifically, BmeSNji and Abr65B significantly increased N, P, Fe, Mn, Zn uptake efficiency (UE) at 30 d and N, K, Zn UE at 60 d. Abr65B also significantly increased Cu UE at 30 d and P UE at 60 d, while BveGB03 could significantly increase Mn, Zn UE at 30 d and N, Zn UE at 60 d (Figure 15).

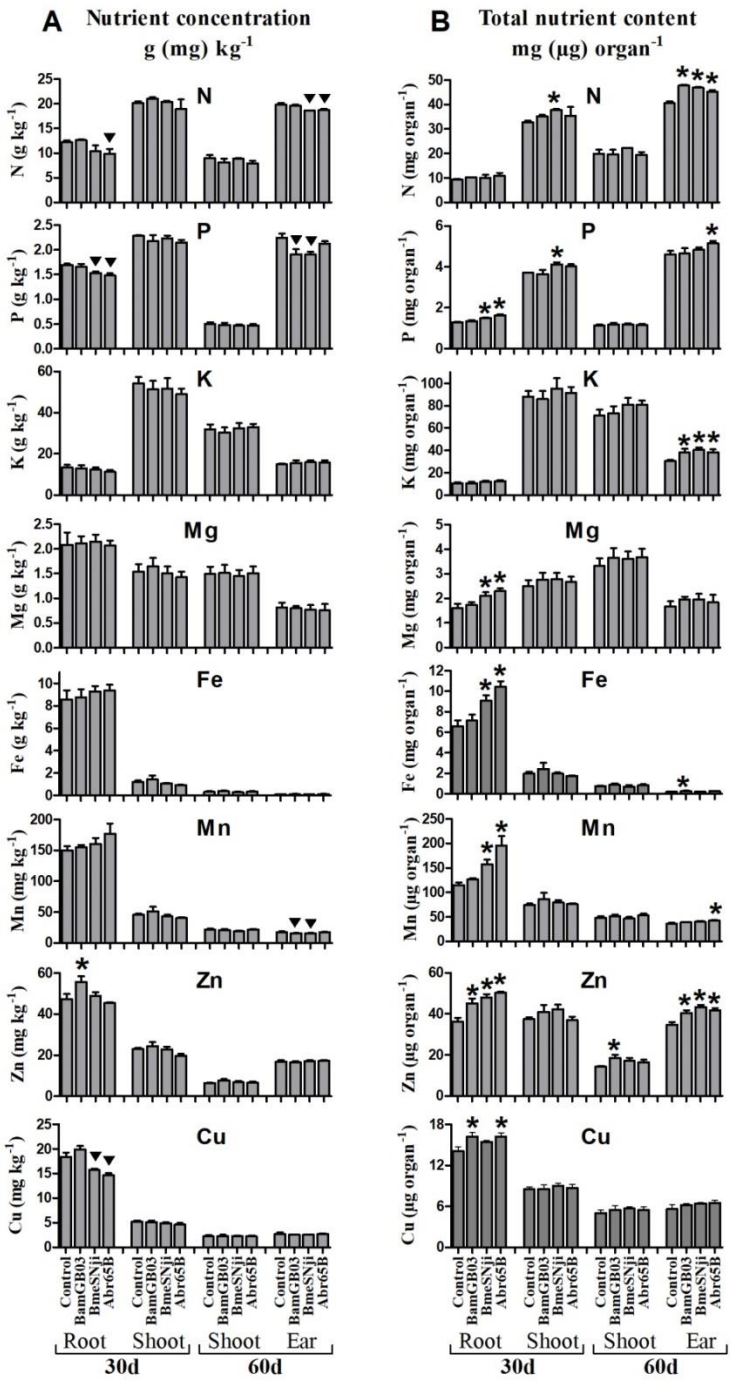


Figure 14– Effects of *B. velezensis*… (continued on the following page)

Figure 14. Effects of *B. velezensis* GB03, *B. megaterium* SNji, and *A. brasilense* 65B inoculations on macro- and micro-nutrient uptake in wheat, 30 d and 60 d after sowing.The nutrient uptake was expressed in terms of (A) nutrient concentrations in different organs (g or mg per kg) and (B) total nutrient contents of the different organs (mg or µg). The 30 d roots and shoots, and 60 d shoots and ears of plants supplied with 50N were used for nutrient analysis due to their high increases in biomass caused by PGPR inoculation. The root samples after 60 d were not used for this analysis due to the aging of roots at mature stages (Zadoks 70) preventing adequate sampling. The values are the means of three replicates ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for each organ. ▼ Significant decrease with $p < 0.05$, * Significant increase with $p < 0.05$.

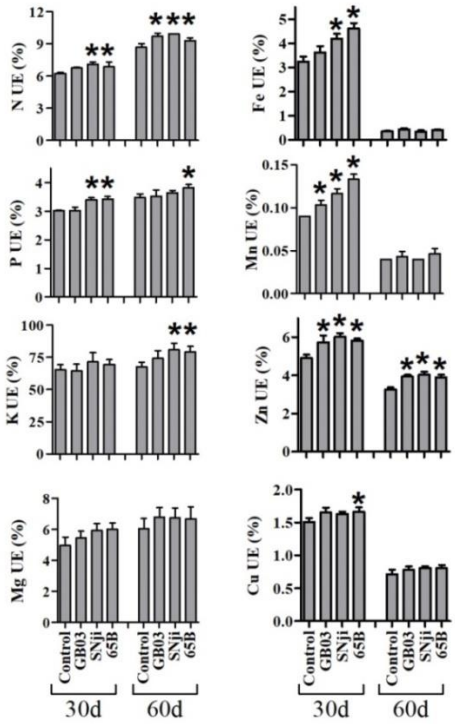


Figure 15. Effect of *B. velezensis* GB03, *B. megaterium* SNji, and *A. brasilense* 65B on nutrient uptake efficiency (UE). N uptake efficiency (N UE) was expressed as the ratio between total N content of the plant per the sum of applied N (50N) and total N available in soil, while the nutrient uptake efficiencies of other elements were expressed as the ratio between the total nutrient content of the plant per the total nutrient available in soil. Plants were grown in soil I and supplied with 50N, then the roots and shoots at 30 d, and shoots and ears at 60 d were used for total nutrient content measurements. The root samples after 60 d were not used for this analysis due to the decaying of roots at mature stages preventing adequate sampling. The values are the means of three replicates ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for each group at 30 d or 60 d (*$p < 0.05$)

4.4 Discussion

4.4.1 PGPR significantly impacted early development of plants under gnotobiotic conditions

Three PGPR strains were able to colonize wheat roots and rhizosphere, and significantly increased the root and shoot biomass of plants grown in sterilized soil under gnotobiotic conditions. This effect was shown to be similar among the three strains, independent of the applied N rates. One possible reason for this observation is the dependence of young seedling growth on its own reserves during the first days of germination. In addition, soil N content was expected to be sufficient to sustain their growth for two weeks during seedling establishment. An increase in plant biomass has also been reported for seven-day-old wheat seedlings inoculated with *P. fluorescens* and *Pantoea agglomerans* in gnotobiotic conditions using sand only (Egamberdieva 2010).

In our study, the growth promotion effect on the early development of seedlings with the inoculation of three strains indicated that they interact with plant processes in other ways than simply by enhancing nutrition. Hormone-like activities are possible mechanisms which were determined in our study. IAA production has been considered as the most important trait of plant growth promotion via the induction of root morphological changes (Duca et al. 2014). By inducing root morphological changes via the auxin signaling pathway, PGPR have the capacity to increase total root area, and hence increase nutrient uptake efficiency and finally plant productivity (Spaepen et al. 2008; Hungria et al. 2010; Zahid et al. 2015). By using mutants of PGPR producing significantly lower IAA amounts, bacterial IAA production has been proven to play a key role in root morphological and physiological changes (Dobbelaere et al. 1999; Idris et al. 2007; Spaepen et al. 2007; Spaepen et al. 2008). In our study, all three strains are able to produce IAA in liquid culture (Table 5). However, the regulation of IAA production in PGPR in a liquid culture is very different from that in real soil under gnotobiotic and greenhouse conditions, which is influenced by various environmental factors (Spaepen et al. 2007; Spaepen & Vanderleyden 2011)**,** such as soil properties (soil texture, spore space, humidity, pH, nutrient status, temperature), root exudates, and complex interactions with the soil microbial community.

Table 5. IAA production capacity of *Bacillus velezensis* GB03, *B. megaterium* SNji, and *Azospirillum brasilense* 65B strains in TSB liquid culture supplemented with or without tryptophan (1g L^{-1}). IAA concentrations are expressed in µg mL^{-1} or pg CFU^{-1}. The values are the mean of six replicates ± SD.

PGPR	- Tryptophan			+ Tryptophan		
	24 h	48 h	72 h	24 h	48 h	72 h
µg mL^{-1}						
BveGB03	9.7 ± 1.6	8.7 ± 1.0	15.3 ± 3.2	6.3 ± 1.8	11.3 ± 0.4	23.4 ± 0.6
BmeSNji	3.0 ± 0.5	4.6 ± 0.4	8.9 ± 0.7	3.7 ± 0.5	6.0 ± 2.1	18.5 ± 4.9
Abr65B	2.1 ± 0.5	3.5 ± 0.2	7.2 ± 0.4	5.2 ± 0.5	15.3 ± 1.8	24.4 ± 3.3
pg CFU^{-1}						
BveGB03	0.13 ± 0.02	0.12 ± 0.01	0.41 ± 0.08	0.08 ± 0.02	0.15 ± 0.02	0.46 ± 0.01
BmeSNji	0.30 ± 0.05	0.24 ± 0.02	0.30 ± 0.02	0.29 ± 0.04	0.28 ± 0.05	0.57 ± 0.15
Abr65B	0.07 ± 0.02	0.11 ± 0.01	0.14 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.11 ± 0.02

4.4.2 Moderate N supply enhanced the plant growth-promoting activity of PGPR at both tillering and flowering stages

Overall, the results from the experiments in soil I and in soil II showed a similar trend, namely that Abr65B was the best strain in promoting wheat productivity, followed by BmeSNji and BveGB03. Both experiments also consistently showed that the highest plant growth-promoting impacts of PGPR were obtained at a moderate N rate, followed by maximal N, and lowest in the absence of N fertilizer addition at both tillering and flowering stages. In contrast, a meta-analysis of 59 studies from 1981 to 2008 (Veresoglou & Menexes 2010) summarized that the maximum plant growth-promoting effects of *Azospirillum* spp. on wheat growth were usually obtained in the absence of any N fertilizer supply, and this was confirmed by other studies (Shaharoona et al. 2008; Hussain et al. 2016). We found only two exceptions under greenhouse conditions, in which the highest *Azospirillum* effect was observed at a high N rate, followed by medium N, with no effect at a low N rate (Millet & Feldman 1984), while another study showed that the *Azospirillum* effect was most pronounced at low-to-intermediate N fertilization rates (Dobbelaere et al. 2002).

However, it is worth noting that the basal, 'low N' level in the absence of fertilizer supply might already be relatively high, depending on soil characteristics, hampering cross-comparison of independent studies. Such a high basal level can result in a good performance of PGPR even with low-N fertilizer supplies, as exemplified in pot trials (Shaharoona et al. 2008). Differences in the performance of PGPR at different N rates between studies might also be explained by the use of different plant cultivars, PGPR strains, growing substrates, growth conditions, and the complex interplay between soil edaphic factors and soil microbial communities. Likewise, the performance of the three strains used in this study, inoculated in non-sterile soil under greenhouse conditions, was not completely consistent with the positive results obtained in autoclaved soil in Magenta boxes, mainly due to their interactions with soil microbial communities and abiotic factors (variable temperature, humidity, air exchange, and light intensity) in the greenhouse.

4.4.3 PGPR counteracted the inhibition of root growth caused by high N supply

The supply of excess nitrate causes a pronounced delay in the elongation of lateral roots after their emergence from parental roots (Zhang et al. 1999). In Arabidopsis, high external nitrate concentrations inhibit lateral root growth

(Hermans et al. 2011). Additionally, wheat does not require a high N supply at the early growth stage; only 30% of the supplied N fertilizer is taken up at the beginning of stem elongation, compared to 90% at flowering (Sylvester-Bradley et al. 2009). We also observed that application of the full N rate to N-rich soil II (Table 4) significantly reduced root biomass in 30-day plants, but Abr65B and BmeSNji inoculations were able to counteract this effect (Fig. 13A, B). Similarly, the inoculation with *Phyllobacterium* restored the inhibition of lateral root development caused by high nitrate supply in *Arabidopsis* grown *in vitro* (Mantelin et al. 2006). By using knockout mutants, Kechid et al. (2013) demonstrated that two nitrate transporter genes – *NRT2.5* and *NRT2.6* – were involved in the lateral root response to the STM196 strain, independently of the N status. Besides, some PGPR use nitrate as an N source for their growth, which potentially decreases the nitrate concentration in the rhizosphere and relieves the inhibition caused by the high N level (Mantelin & Touraine 2004). To the best of our knowledge, this study provides the first evidence that PGPR can mitigate root growth inhibition caused by high N availability under realistic soil conditions. We hypothesize that inoculation with PGPR could be useful for enhancing N uptake, by sustaining root branching in conditions of high N supply, resulting in less N lost by leaching or volatilization.

4.4.4 PGPR enhanced total nutrient content rather than nutrient concentration

Many studies on the effect of PGPR inoculation on nutrient uptake have expressed their impact in terms of nutrient concentration and/or total nutrient content of plants or plant organs. In our work, all strains resulted in similar, significantly lower nutrient concentrations in shoots, roots, or ears as compared to the non-inoculated controls, except for Zn in roots inoculated with BveGB03. In our conditions, high biomass increases by PGPR treatments correlates with lower nutrient concentrations, particularly in roots (with an increase up to +45% in 30 d plants) and in ears (up to +23% in 60 d plants). We hypothesize that this observation may be explained by some '*dilution*' effect, whereby the biomass increase due to PGPR inoculation exceeds the enhancement of nutrient uptake. Dobbelaere et al. (2002) found that, in most cases, the N concentration of wheat inoculated with *A. brasilense* was unchanged. The study by these authors was limited to a plant stage of 1 week after flowering, however, and only N was included. Hungria et al. 2010 also found an unchanged concentration of all macro- and micro-nutrients in wheat leaves at the flowering stage, but in another replicate the nutrient concentrations in inoculated plants varied, being both lower and higher than those of the control.

However, how much wheat leaf biomass was promoted by inoculants was not mentioned in the study. Therefore, we have attempted to draw a global picture including most of the common macro- and micro-nutrients in combination with different plant parts (root, shoot, ear) and different plant stages (30 d and 60 d) for which high growth stimulation by the PGPR was observed.

Regarding the peculiar response of Zn, inoculation with endophytic strains (of *B. subtilis* and *Arthrobacter*) has been shown to result in an increased Zn concentration in wheat, possibly related to bacterial IAA production (Singh et al. 2017). In our study, the three strains were able to solubilize insoluble phosphate at various levels (according to the strains) in a liquid culture medium (Figure 16), but none of them resulted in any significant enhancement of P concentration in inoculated plants grown in soil in the greenhouse.

In contrast to nutrient concentration, the total nutrient content was in general increased by inoculation, probably due to the increased biomass. This is in agreement with several other studies where an increase in macro-nutrients (e.g., N content; see Saubidet et al. 2002; Adesemoye et al. 2010) or micro-nutrients (e.g., Fe, Mn, and Zn; see Ogut & Er 2016; Singh et al. 2017) is observed. In general, we observed a higher increase for micro-nutrients compared to macro-nutrients in inoculated plants, corroborating results observed by (Ogut & Er 2016). All three tested PGPR strains in this study were able to increase total N content, and hence improve N uptake efficiency in plants, confirming previous studies (Spaepen et al. 2008; Ahmad et al. 2017).

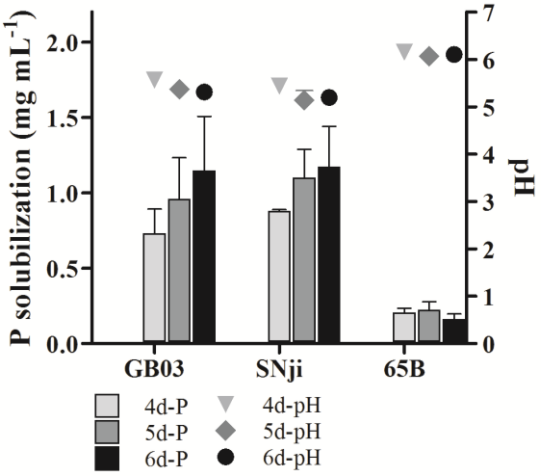


Figure 16. Phosphate solubilization capacity of *Bacillus velezensis* GB03, *B. megaterium* SNji, and *Azospirillum brasilense* 65B strains in liquid culture (NBRIP medium) containing 0.5% insoluble phosphate $Ca_3(PO_4)_2$. Bacteria were added to the medium with a final cell density of 10^7 CFU mL^{-1}. After 4, 5, and 6 days incubation, solubilized P and pH were measured. The values are the mean of three replicates ± SD.

4.5 Conclusions

PGPR have been proposed as a tool to reduce fertilizer inputs while maintaining high yields, due to their capacity to increase root growth and nutrient uptake (Dobbelaere et al. 2002; Adesemoye & Kloepper 2009; Le Mire et al. 2016). Our work indicates that N fertilizers and PGPR interact in their effects on plant growth and that the highest growth benefits of PGPR inoculation are not necessarily observed with the lowest rates of N fertilizers. Regarding plant stages, the biostimulant effects of PGPR can be clearly observed in roots during early plant development under gnotobiotic conditions, and biostimulation was also observed in roots at the end of tillering to the beginning of stem-elongation stage, or in ears at the flowering stage of plants grown in greenhouse. Higher uptake efficiency of fertilizers caused by PGPR inoculation does not necessarily result in higher tissue concentrations of the nutrients, which can even be lowered by the PGPR when compared with the non-inoculated control.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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5

Biostimulant effects of *Bacillus* strains on wheat from *in vitro* towards field conditions are modulated by nitrogen supply

5 Biostimulant effects of *Bacillus* strains on wheat from *in vitro* towards field conditions are modulated by nitrogen supply

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Abstract

Bacillus velezensis strains belonging to plant growth-promoting rhizobacteria (PGPR) are increasingly used as microbial biostimulant. However, their field application on winter wheat under temperate climate remains poorly documented. Therefore, three *B. velezensis* strains, IT45, FZB24, and FZB42 were tested for their efficacity under these conditions. Two biological interaction systems were firstly developed under gnotobiotic and greenhouse conditions combined with sterile or non-sterile realistic soil, respectively, and finally assayed in the field during two years coupled with different N fertilization rates. Under gnotobiotic conditions, all three strains significantly increased root growth of 14-day-old spring and winter wheat seedlings. In the greenhouse using non-sterile soil, only FZB24 significantly increased root biomass of spring wheat (+31%). The three strains were able to improve nutrient uptake of the spring wheat grown in the greenhouse, particularly for micronutrients Fe, Mn, Zn, and Cu, but the observed increases in nutrient uptake were dependent on the organs and the elements. The root biomass increases in inoculated plants went along with lowered nutrient concentrations of P and K. In 2014, under field conditions and absence of any N fertilizer supply, FZB24 significantly increased grain yields by 983 kg ha^{-1}, or 14.9%, in relation to non-inoculated controls. The three strains in the 2015 field trial failed to confirm the previous positive results, likely due to the low temperatures occurring during and after inoculations. The Zeleny sedimentation value, indicative of flour quality, was unaffected by the inoculants. The results are discussed in the perspective of bacterial application to wheat under temperate agricultural practices.

Key words: PGPR, winter crop, nutrient uptake, nitrogen fertilizer, spraying inoculation, plant stage

5.1 Introduction

Bacillus velezensis [some strains being referred as *B. amyloliquefaciens* or *B. subtilis* in the past; Dunlap et al. (2016), Fan et al. (2017), Wang et al. (2008)] is considered to be a plant growth-promoting rhizobacterium (PGPR) (Idris et al., 2007, 2004; Yao et al., 2006) and belongs to one of the main categories of biostimulants (du Jardin, 2015). Plants inoculated with *Bacillus* show improved root growth, as well as total root area, and consequently enhanced nutrient uptake efficiency which is often low in crops (Adesemoye & Kloepper 2009). Such enhanced root growth and altered root morphology can be induced by phytohormones and/or analogs produced by PGPR, such as cytokinins, indole-3-acetic acid (IAA), gibberellins, reducing ethylene production by ACC deaminationa (Duca et al. 2014), of which IAA might play a key role (Spaepen et al. 2008). Besides, *B. velezensis* is able to form heat- and desiccation-tolerant endospores (Berendsen et al. 2016) which facilitate the formulation process as well as extend its shelf-life (Bashan et al. 2014). Therefore, strains belonging to *B. velezensis* have commonly been selected for formulation as biostimulant and/or biocontrol products (Le Mire et al. 2016).

Wheat (*Triticum aestivum* L.) is one of most important cereal crops. It is successfully grown between the latitudes of 30–60°N and 27–40°S. Winter wheat cultivars are commonly grown in Belgium due to their good adaptation to the oceanic temperate climate. The grain yield values of 8.0–9.5 tons ha^{-1} are among the highest values in the world (Top 10 wheat yield, FAO 2012). However, the number of PGPR application studies on improving the winter wheat productivity in Belgium as well as other temperate climate regions is very scarce, and such field experiments were mainly carried out before 2001 (Reynders & Vlassak 1982; de Freitas & Germida 1992; Germida & Walley 1996; Swędrzyńska 2000; Dobbelaere et al. 2001a). *Pseudomonas*, *Azospirillum*, and *Azotobacter* strains were mainly used in such studies. To the best of our knowledge, there has not been any study assessing the impact of *Bacillus*, which is an important genus within the PGPR group, on grain yield of winter wheat under field conditions in temperate regions.

It has always been questioned whether the positive results on plant growth induced by PGPR under greenhouse conditions can be reproduced under field conditions (Veresoglou & Menexes 2010). Therefore, we aimed answering the following questions:

(1) Is the plant growth-promoting effect of *B. velezensis* reproducible in different cultivated systems, i.e. from close-air gnotobiotic conditions, over open-air conditions of greenhouse towards field conditions?

(2) Do *B. velezensis* strains influence the nutrient uptake of wheat at young stages?

(3) Do *B. velezensis* strains affect the productivity of winter wheat under field conditions following Belgian conventional crop management practices?

To achieve the objectives, experiments were designed using three *B. velezensis* strains, IT45, FZB24, and FZB42, inoculated to spring and winter wheat cultivars under gnotobiotic conditions using sterile soil, greenhouse conditions using non-sterile soil, and field conditions using winter wheat coupled with different N fertilizer rates.

5.2 Materials and methods

5.2.1 *Bacillus velezensis* strains

Three strains, *B. velezensis* IT45 (Rhizocell GC, Ithec, Lallemand Plant Care SAS, France; formerly referred to as *B. amyloliquefaciens / subtilis* IT45; Fan et al., 2017), *B. velezensis* FZB24 (Abitep GmbH, Germany; formerly referred to as *B. amyloliquefaciens / subtilis* FZB24; Fan et al., 2017) and *B. velezensis* FZB42 (Abitep GmbH; formerly referred to as *B. amyloliquefaciens / subtilis* FZB42; Fan et al., 2017) were used for the experiments. The bacterial concentration of IT45 ($\simeq 10^9$ CFU g^{-1}, CFU: colony-forming unit), FZB24, and FZB42 ($\simeq 2.5\times10^{10}$ CFU mL^{-1}) were confirmed by plating in serial 10-fold dilutions on tryptic soy agar medium (TSA, Sigma-Aldrich, Missouri, US). The three strains were selected from preliminary screenings of six commercial rhizobacteria under greenhouse conditions (as presented in the "Greenhouse experiments" section) based on their plant biomass promoting capacity (Table 3, Figure 5).

5.2.2 Soil preparation for Magenta box and pot cultivation

A field soil was favored over commercial composts, perlite, or sand to ensure a realistic rooting medium as close as the one observed in the field. Top soil layer (0–30 cm depth) samples were collected from Bordia, Gembloux, Belgium (50°34'05.8"N, 4°42'33.0"E). The soil contained 22% clay, 69% silt, 9% sand; total N was 1.02 g kg-1 soil, NO3- 5.9 mg kg-1, pH KCl 7.2; 100 g soil contained 1.13 g C organic total, 22.0 mg P, 15.0 mg K, 11.0 mg Mg, 240 mg Ca; 1 kg soil contained 2.0 mg Cu, 2.0 mg Zn, 352.0 Fe, 273.0 Mn (see "Soil physicochemical and plant nutrient analysis" section). Three volumes of dried soil were mixed with one volume of dried sand in a cement mixer machine in order to partially restore the soil macroporosity. This soil mixture was used for both Magenta box and greenhouse

cultivation. In order to prepare autoclaved soil for Magenta box cultivation, thin layers (less than 2 cm) of soil mixture were deposited in trays, covered by aluminum foil, and autoclaved three times at 121 °C for 1 h on alternate days with overnight incubation in a laminar flow cabinet in between.

5.2.3 Gnotobiotic experiments in Magenta boxes

Grains of spring wheat (*Triticum aestivum* L. cv. Tybalt) and winter wheat (*T. aestivum* L. cv. Forum) were surface sterilized in 70% ethanol (30 s) then rinsed 5 times with sterile water. They were subsequently incubated in $AgNO_3$ (1% w/v) at 200 rpm for 20 min, then rinsed 5 times with sterile water. Next, the grains were treated with 3% hypochlorite (NaClO) solution for 20 min at 200 rpm, and then rinsed 5 times with sterile water before incubation in sterile water for 30 min, at 200 rpm. Grains were germinated on wet sterile filter paper in Petri dishes at room temperature in the dark for 36 h. The grains were finally dipped in 10^8 CFU mL^{-1} bacterial suspension (Çakmakçı et al., 2014; Nguyen et al., 2018; Salantur et al., 2006; Shaharoona et al., 2008; Yegorenkova et al., 2016) in phosphate buffer for 30 min and five germinating grains were transferred to each Magenta box (size GA-7; 75 mm × 75 mm × 97 mm, Sigma Chemical Co., St. Louis, MO). The sterile phosphate buffer was used as control. The two Magenta chamber halves were coupled together with a plastic collar (75 mm × 75 mm × 20 mm) and then filled with 100 g of autoclaved soil. The Magenta boxes were placed in a growth chamber with a 16 h photoperiod with an average PPFD of ≃150 µmol $m^{-2}s^{-1}$ and a mean temperature of 23 °C.

The PGPR colonization in roots and rhizosphere soil was assessed with a modified protocol from (Chowdhury et al., 2013). Loosely adhering soil was removed by hand-shaking from the roots and the remaining soil surrounding the roots was considered as rhizosphere soil. Three grams of fresh roots and rhizosphere soil were suspended in 10 ml sterile phosphate-buffered saline (NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.15 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, pH 7.3) in 100 ml sterile Erlenmeyer flasks containing 15 glass beads (0.6 mm in diameter) and shaken vigorously for 1h at 300 rpm. Afterwards, the suspension was plated in serial 10-fold dilutions on TSA medium and total colonies were then counted after 24–48h.

5.2.4 Greenhouse experiments

The pot cultivations were conducted in the greenhouse at the Plantes-Systèmes Facility, Gembloux Agro-Bio Tech. Spring wheat (*T. aestivum* cv. Tybalt) grains

were germinated on moist filter papers for two days without surface sterilization. Five germinating grains were sown in a pot made with a cut PVC tube (30 × 9 cm) filled with 2 kg of the soil mixture in a plastic bag, and finally thinned into two homogenous plants per pot four days after sowing. One mL of bacterial suspension (1×10^8 CFU mL^{-1} phosphate buffer) was inoculated to each 4-day-old plantlet and re-inoculated every two weeks with the same concentration. The sterile phosphate buffer was used as control. The humidity of soil in pots was maintained at 13-15% (w:w). A 16 h photoperiod was applied with an average PPFD of ≃170 µmol $m^{-2}s^{-1}$ (daylight in greenhouse supplemented with LED light, Vegeled Flood Light BX151, Colasse SA, Seraing, Belgium). The first test was performed in May 2014 while average temperature in greenhouse was 23.2 ± 2.5 °C and the second test took places in October 2014 with 21.9 ± 0.2 °C. The overall experiments were designed in completely randomized blocks with eight replicates per treatment.

5.2.5 Field experiments

In the 2013–2014 field trial, grains of winter wheat (*Triticum aestivum* L. cv. Forum) were sown in Bordia, Gembloux, Belgium (50°34'08.4"N 4°42'38.6"E, which belongs to the oceanic temperate region) on 2 December 2013 with density of 400 grains m^{-2}. The 2014–2015 field trial was carried out in Lonzée, Gembloux (50°33'18.1"N 4°44'31.3"E) and grains were sown on 14 October 2014 with a density of 250 grains m^{-2}. Plot size was 16 m^2 (8 × 2 m) and five replicates (plots) were used per treatment. The plots were randomized in criss-cross design in five main blocks with PGPR and N rates treatments as sub-blocks. In 2013–2014, fungicides Osiris (BASF Crop Protection Belgium, 1 L ha^{-1}) and Aviator Xpro (Bayer Cropscience, 1.25 L ha^{-1}) were applied on 5 May at the last leaf stage and on 6 June 2014 during flowering, respectively. In 2014– 2015, the fungicides were applied on 4 May and on 9 June 2015, respectively. Growth reducer (Meteor, BASF Crop Protection, 2L ha^{-1}) was sprayed in April 2014 and 2015 to prevent lodging.

In the first field trial, the bacterial products were sprayed on 24 April (Zadoks 31) and on 23 May 2014 (Zadoks 39). In the second field trial, they were sprayed on 26 March (Zadoks 30) and on 29 April 2015 (Zadoks 35–37). Those spraying times were selected to fit between the split N applications to maximize the N uptake in plants (Figure 17). The inoculants were mixed with water (400L ha^{-1}) for spraying. The spraying times were selected between moderately (lower than 5mm) rainy days to support the bacterial mobility without drenching out the inoculants. The inoculant doses were defined according to the manufacturers recommendations [2×10^8 CFU m^{-2} for IT45 (1kg ha^{-1}), 2×10^{10} CFU m^{-2} for both FZB24 and FZB42 (0.04 % for

drenching or 0.8 mL m²)]. In the 2015 field trial, besides the same experimental sets as in 2014 trial, FZB24 and FZB42, which have high bacterial concentration (2.5×10^{10} CFU mL^{-1} solution), were normalized to 5×10^{10} CFU m^2 as in Warembourg et al. (1987).

In the 2014 field trial, 100% N fertilization dose (ammonium nitrate; 27%, Brichart S.A.) was applied at 175 kg N ha^{-1} (i.e. 648.2 kg ammonium nitrate ha^{-1}), in which 50 kg N ha^{-1} were applied at tillering, 50 kg N ha^{-1} at stem elongation, and 75 kg N ha^{-1} at the last leaf stage. In the 2015 trial, 100% N was 185 kg N ha^{-1}. The same split N ratios were applied for 75% N and 50% N. Ear number was counted in three wheat lines with 0.5 m length each line and 10 replicates. Grain yields were harvested by a combined harvester and the values are means of five plots. 1000 seed weight and Zeleny sedimentation value, indicative of flour quality, were also measured with five replicates.

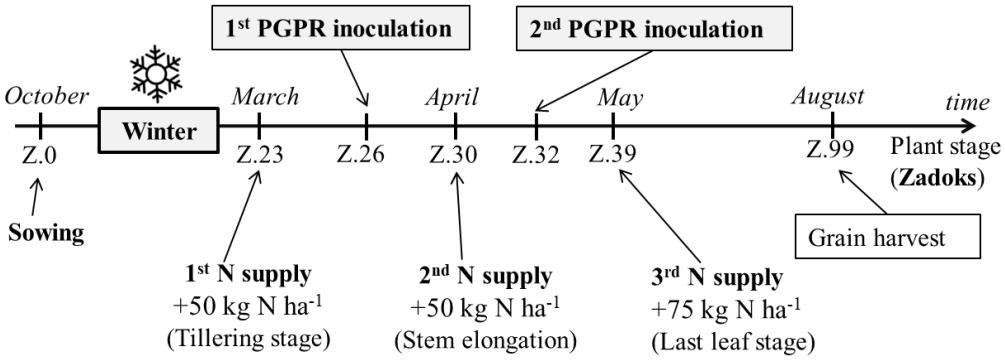


Figure 17. Timeline of the field trials with repeated PGPR inoculations and N fertilizer application according to wheat developmental stages. The PGPR inoculation times were selected to fit between the split N applications to maximize the N uptake in plants.

5.2.6 Soil physicochemical and plant nutrient analysis

Air-dried soil samples were sieved at 2 mm and ground to 200 μm. Particle size of sand, silt, and clay fractions was determined (AFNOR NF X31-107), as well as soil pH (1N KCl, ISO 10390-2005), total organic carbon (Walkley and Black method, modified ISO 14235-1998), total N (Khjeldahl method, ISO 11261-1995), and soil NO_3^- (QuickChem® method, 12-107-04-1-B). After extraction with CH_3COONH_4 (0.5 M) and EDTA (0.02 M) at pH 4.65 (w:v 1:5 ratio), elemental

concentrations were quantified by colorimetry for P (at 430 nm), and flame atomic absorption spectrometry (VARIAN 220, Agilent Technologies, CA, USA) for K, Mg, and Ca.

Plant samples were ground into powder, and 2 to 5 g of the powder were digested in a mixture of concentrated HNO_3 and $HClO_4$ (15ml of mixture for each g of the powder). Next, the element concentrations were quantified by colorimetry for P and flame atomic absorption spectrometry for the other elements. The values are the mean of three technical replicates from a pool of eight plants.

Table 6. Mean values of soil physical and chemical properties in the top 30 cm–layer in Bordia and Lonzée (Belgium) for the field trials in 2014 and 2015, respectively

Year	Previous crop	Clay	Silt	Sand	pH KCl	Total organic C ($g\ 100g^{-1}$)	$N\text{-}NO_3$ ($mg\ kg^{-1}$)	P	K	Mg	Ca
		%						$mg\ 100g^{-1}$			
2014	Oil rape	22	69	9	7.15	1.48	5.86	22.3	29.3	14.5	309
2015	Sugar beet	24	68	8	7.31	1.19	5.58	9.5	17.2	9.3	369

5.2.7 Colorimetric determination of indole-3-acetic acid (IAA) in liquid culture

IAA production capacity of PGPRs was measured by colorimetric method (Sarwar et al. 1992) without adding tryptophan. The PGPR strains were cultured in TSB liquid medium (tryptic soy broth, Sigma-Aldrich, Missouri, US) at 30 °C and the IAA concentrations were measured after 24, 48, and 72 h.

5.2.8 Statistical analysis

The data of root and shoot biomass of plants in Magenta box and pot experiments were analyzed using one-way ANOVA, followed by Dunnett's Multiple Comparison Test (α=0.05). The data of grain yield, ear number, and Zeleny parameter of both field trials were analyzed using two-way ANOVA with 2 factors, inoculations (3 PGPRs and control) × N rates (0, 50, 75, 100N), followed by Dunnett's Multiple Comparison Test (α=0.05). These analyses were performed using GraphPad Prism 7.03 (GraphPad Software Inc., Suite 230 La Jolla, CA, USA).

5.3 Results

5.3.1 IAA production of PGPRs in liquid culture

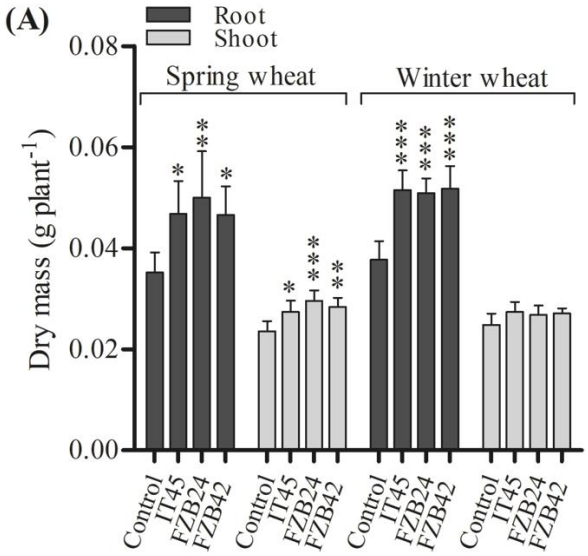
The *B. velezensis* strains were able to produce IAA in TSB liquid culture (Table 6). They produced higher IAA values at 72 h than that at 48 h and 24 h, therefore only the results at 72 h are presented. In our study, IT45, FZB24, and FZB42 produced 20, 36, and 29 µg IAA mL^{-1}, or 0.5, 2.2, and 0.9 pg IAA CFU^{-1}, respectively.

Table 7. IAA production by *B. velezensis* strains in TSB culture medium after 72 h. The IAA production capacity is expressed in µg mL-1 or pg CFU-1. Each value represents the mean of six replicates ± SD**.**

PGPR strain	Bacterial IAA production		CFU mL^{-1}
	µg mL^{-1}	pg CFU^{-1}	
IT45	19.46 ± 1.64	0.49 ± 0.04	$7.60 \pm 0.15 \times 10^7$
FZB24	35.78 ± 9.74	2.18 ± 0.59	$7.21 \pm 0.36 \times 10^7$
FZB42	29.32 ± 8.10	0.90 ± 0.25	$7.51 \pm 0.40 \times 10^7$

5.3.2 Gnotobiotic trials

The effects of *B. velezensis* inoculations on wheat growth seem to be cultivar-specific under gnotobiotic conditions (Figure 18A). Regarding spring wheat, IT45, FZB24 and FZB42 significantly increased 14-day-old root dry biomass (by 33, 42 and 33%, respectively, as compared to the non-inoculated control), and shoot dry biomass (by 16, 26 and 20%, respectively). In the case of winter wheat, all three strains were also able to significantly increase root dry biomass (by 37, 35, and 37%, respectively), but there was no significant increase in shoot biomass of inoculated plants. The three *Bacillus* strains colonized the roots and rhizosphere of spring and winter wheat with densities of $5.1–5.7 \times 10^6$ CFU g^{-1} fresh root and rhizosphere for IT45, 2.3×10^7 for FZB24, and $1.5–1.8 \times 10^7$ for FZB42 14 days after seed inoculation (Figure 18B).



(B) Root colonization (CFU g^{-1} root and rhizosphere)

Treatment	Spring wheat	Winter wheat
Control	0	0
IT45	$5.11 \pm 0.34 \times 10^6$	$5.67 \pm 0.37 \times 10^6$
FZB24	$2.25 \pm 0.19 \times 10^7$	$2.30 \pm 0.45 \times 10^7$
FZB42	$1.81 \pm 0.42 \times 10^7$	$1.49 \pm 0.56 \times 10^7$

Figure 18. Effects of *B. velezensis* inoculation on early development of spring and winter wheat grown in sterile soil under gnotobiotic conditions. (A) Dry root and shoot biomasses of spring wheat (on the left) and winter wheat (on the right) grown in Magenta boxes after 14 days. (B) Colonization of *B. velezensis* in roots and rhizosphere after 14 days. The experiments were repeated twice with similar results (only one replicate is presented). The values are means of 6 boxes ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test (α=0.05) was applied for each group of root or shoot values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5.3.3 Greenhouse trials

The greenhouse trials in May 2015 showed that FZB24 was able to significantly increase root biomass by 31% as compared to the non-inoculated control (Figure 19). IT45 and FZB42 also promoted root growth by 17% without any statistically significance. In the second test in October, there was no significant difference in biomass of inoculated plants compared to the control. Only slight increases were observed in root dry biomass by 6% with FZB24 inoculation and shoot dry biomass by 8% with IT45, 11% with FZB24, and 9% with FZB42. The average air temperature in the greenhouse in May and October was 23.2 ± 2.5 °C and 21.9 ± 0.2 °C, respectively.

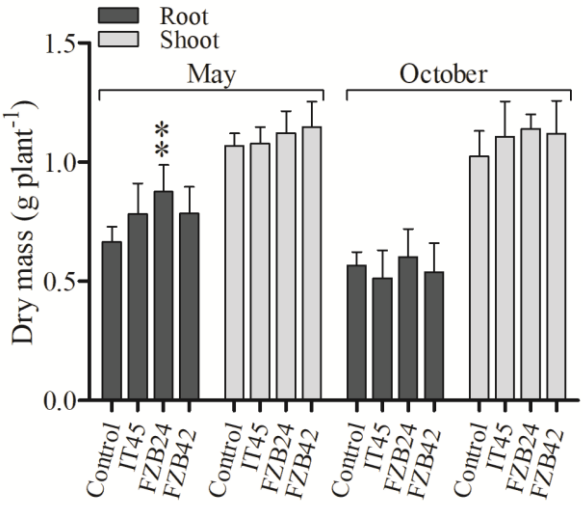


Figure 19. Effects of *B. velezensis* IT45, FZB24, and FZB42 inoculation on spring wheat under greenhouse conditions in May and October 2015. Dry biomasses of root and shoot were measured 30 days after sowing (Zadoks 30-32). The values are the means of 8–10 pots for inoculations and 13–15 pots for controls ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test (α=0.05) was applied for each group of root or shoot. ** $p<0.01$

The nutrient uptake of spring wheat grown in greenhouse was expressed in terms of *nutrient concentration* and *total nutrient content* (defined as the total amount of elements per plant organ) (Figure 20 and 21). The aim was to test if increased biomass promoted by PGPR goes along with increased nutrient uptake. Regarding *macro-nutrient concentrations* (Figure 208A), FZB24 significantly reduced the concentrations of P and K, and the same held true for IT45 for K in roots, while N

concentration was significantly increased in shoots by FZB42 treatment. In contrast, *micro-nutrient concentration* (Figure 21A) generally increased or remained unchanged in both roots and shoots of inoculated plants. Specifically, FZB24 significantly increased Fe, Mn concentrations in roots, and Fe, Mn, Zn, Cu in shoots, while IT45 increased Fe and Mn in shoots only. In term of *total nutrient content*, however, the total element contents were generally increased or unchanged (Figure 20B and Figure 21B). Particularly in roots, FZB24 significantly increased the total contents in N, Mg, Fe, Mn, Zn, and Cu; the same held true for IT45 with Fe. In shoots, FZB24 significantly increased the total contents in P, K, Fe, Mn, Zn, and Cu; so did IT45 with Fe and Mn, and FZB42 with N, Zn, and Cu.

Noticeably, the increase in concentration and total content of micro-nutrients (i.e. Fe, Mn, and Zn) (Figure 21) were more pronounced than that of macro-nutrients (Figure 20) in inoculated plants as compared to non-inoculated controls. For instance, Fe concentration was improved by 26% in roots and 203% in shoots of plants inoculated with FZB24, and by 85% in shoots with IT45. Also, Mn concentration was enhanced by 22% in roots and 40% in shoots with FZB24, and Zn concentration was increased by 22% in shoots with FZB24 inoculation.

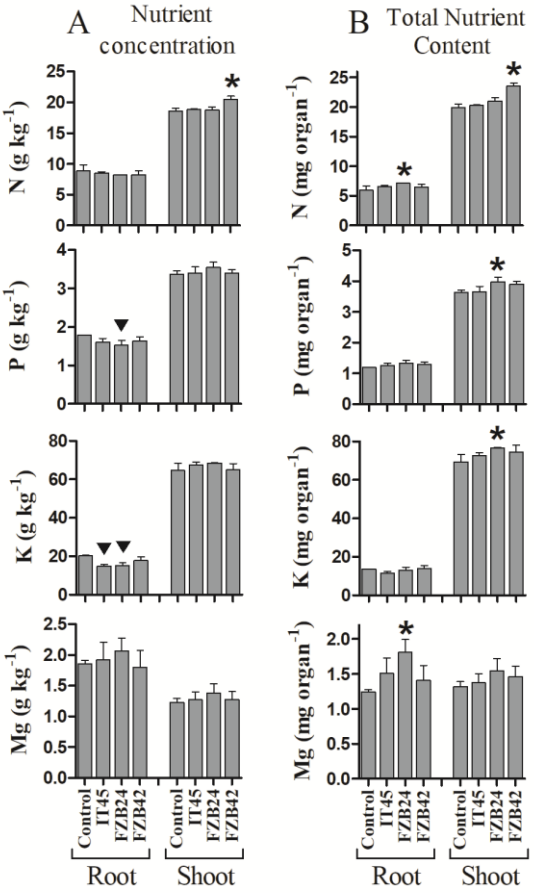


Figure 20. Effects of *B. velezensis* on the main macro-nutrients uptake in spring wheat 30 days after sowing under greenhouse conditions. The nutrient uptake was expressed in terms of (A) nutrient concentration (g or mg per kg dry biomass) and (B) total nutrient content (mg or µg per organ). The spring wheat plants harvested in May 2015 were used for the nutrient analysis. The values were the mean of three technical replicates ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for each organ values.▼ Significant decrease with $p<0.05$, * significant increase with $p<0.05$

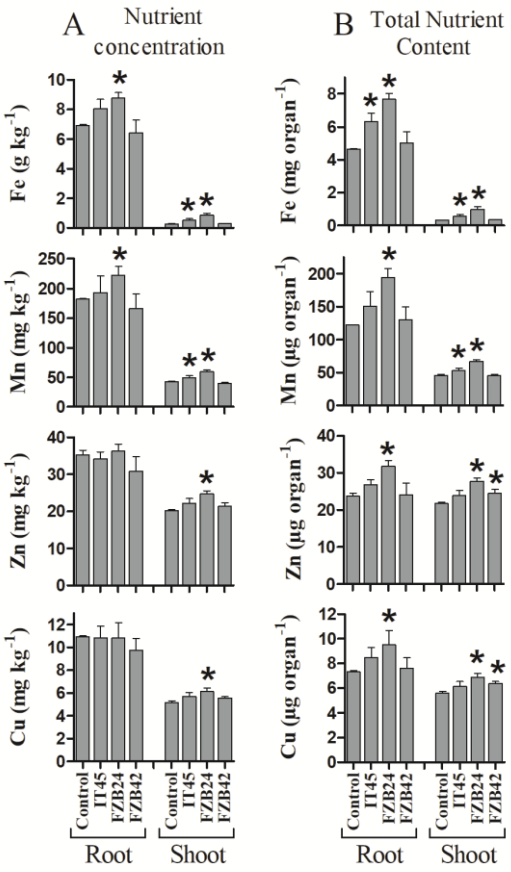


Figure 21. Effects of *B. velezensis* on the main micro-nutrients uptake in spring wheat 30 days after sowing under greenhouse conditions. The nutrient uptake was expressed in terms of (A) nutrient concentration (g or mg per kg) and (B) total nutrient content (mg or µg for each organ). The spring wheat plants harvested in May 2015 were used for the nutrient analysis. The values were the mean of three technical replicates ± SD. One-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for each organ values. *Significant increase with $p<0.05$

5.3.4 Field trials

Two field trials were carried out in 2013–2014 and 2014–2015 at two locations (Bordia and Lonzée respectively), and the soil physical and chemical properties of the two locations are listed in Table 7. Analysis of variance of both field trials showed the grain yield, ear number, and Zeleny parameter displayed a marked response towards different N rates (Table 8 and 9). In the 2013–2014 field trial, the highest increase in grain yield promoted by inoculants compared to the non-inoculated control was +14.9% with FZB24 in the absence of N supply ($p=0.021$) (Table 8). Overall, the highest change in grain yield triggered by IT45, FZB24, and FZB42 inoculation compared to the non-inoculated control was obtained at 0N (+6.0, 14.9, 6.7%, respectively), followed by 50N (+4.4, 6.3, 2.3%). Such changes were lower when higher N rates were supplied at 75N (+2.7, 5.3, 2.4%) and 100N (+3.4, 3.0, 2.7%). Likewise, the maximum increases in ear number and Zeleny parameter were +7.9% and 8.1% respectively with IT45 inoculation at 0N. Noticeably, FZB24 resulted in higher ear number m^{-2} with an increase of 6.8% at 50N, 7.7% at 75N, and 6.4% at 100N. In the 2015 field trial, however, the changes in all of the yield parameters in inoculated plants compared to the non-inoculated control at each N rate were much lower than those of the 2014 field trial (Table 9). The maximum changes in grain yield, ear number, and Zeleny parameter were +4.2%, 9.3%, and 6.5% following inoculations of FZB24–2.5X at 50N, FZB42–2.5X at 0N, and FZB24 at 50N, respectively. Besides, PGPR treatments had no effect on the 1000-seed weight in both field trials ($\simeq$ 50–53 g 1000 $seed^{-1}$ in all treatments, data not shown).

Table 8. Effect of *B. velezensis* on wheat yield and quality traits of winter wheat in the 2013–2014 field trials under different N fertilization rates. The values are means of five replicates for grain yield and Zeleny parameter, and ten replicates for ear number ± SD. Two-way ANOVA followed by Dunnett's Multiple Comparison Test (α=0.05) was applied for each quality trait. * p<0.05

N rates	PGPR	Grain yield (ton ha^{-1})	Ear number (m^{-2})	Zeleny (ml)
0N	Control	6.59	363	14.7
	IT45	6.99 *(6.0)*	391 *(7.9)*	15.9 *(8.1)*
	FZB24	7.58 *(14.9)* *§	373 *(2.8)*	15.3 *(4.1)*
	FZB42	7.04 *(6.7)*	359 *(-0.9)*	15.1 *(2.8)*
50N	Control	8.91	448	21.4
	IT45	9.30 *(4.4)*	442 *(-1.8)*	21.1 *(-1.5)*
	FZB24	9.47 *(6.3)*	481 *(6.8)*	21.0 *(-1.7)*
	FZB42	9.11 *(2.3)*	421 *(-6.0)*	21.1 *(-1.5)*
75N	Control	9.80	485	23.5
	IT45	10.04 *(2.7)*	491 *(0.7)*	22.8 *(-3.3)*
	FZB24	10.30 *(5.3)*	516 *(7.7)*	24.1 *(2.2)*
	FZB42	10.01 *(2.4)*	493 *(2.0)*	23.4 *(-0.5)*
100N	Control	10.36	493	25.1
	IT45	10.71 *(3.4)*	494 *(0.1)*	25.7 *(2.4)*
	FZB24	10.68 *(3.0)*	525 *(6.4)*	25.7 *(2.7)*
	FZB42	10.64 *(2.7)*	485 *(-1.8)*	25.7 *(2.6)*
		(%)	*(%)*	*(%)*
Variables		*p* value		
N Rates		<0.0001	<0.0001	<0.0001
PGPR		0.0198	0.0032	0.81
N × PGPR		0.98	0.57	0.68

(%) relative changes in grain yield and quality traits of inoculated plants compared to the corresponding control at each N rate.

§ FZB24 vs control at 0N: p=0.021, q=2.754. The other p values of other PGPR treatments are always higher than 0.20 (not shown).

Table 9. Effect of *B. velezensis* on wheat yield and quality traits of winter wheat in the 2014–2015 field trials under different N fertilization rates. Besides the recommended concentration (1 X), FZB24 and FZB42 concentrations were increased by 2.5 folds (2.5 X) to reach 5×1010 CFU m^{-2}. The values are means of five replicates for grain yield and Zeleny parameter, and ten replicates for ear number ± SD. Two-way ANOVA followed by Dunnett's Multiple Comparison Test (α=0.05) was applied for each quality trait.

N rates	PGPR	Grain yield (ton ha^{-1})	Ear number (m^{-2})	Zeleny (ml)
0N	Control	5.18	301	14.8
	IT45	5.17 *(-0.1)*	324 *(7.5)*	15.6 *(5.7)*
	FZB24	5.02 *(-3.2)*	293 *(-2.9)*	14.8 *(0.2)*
	FZB24-2.5X	5.23 *(0.9)*	314 *(4.2)*	15.5 *(4.8)*
	FZB42	4.95 *(-4.5)*	316 *(4.9)*	15.6 *(5.5)*
	FZB42-2.5X	5.43 *(4.7)*	329 *(9.3)*	15.3 *(3.9)*
50N	Control	7.98	451	21.5
	IT45	7.93 *(-0.6)*	436 *(-3.3)*	21.6 *(0.5)*
	FZB24	8.08 *(1.2)*	451 *(0.0)*	22.9 *(6.5)*
	FZB24-2.5X	8.32 *(4.2)*	417 *(-7.4)*	21.4 *(-0.5)*
	FZB42	8.11 *(1.6)*	435 *(-3.4)*	21.1 *(-1.6)*
	FZB42-2.5X	8.12 *(1.7)*	435 *(-3.4)*	20.6 *(-4.1)*
100N	Control	10.00	526	29.1
	IT45	10.06 *(0.6)*	526 *(0.0)*	27.9 *(-4.0)*
	FZB24	10.11 *(1.0)*	511 *(-2.9)*	28.2 *(-3.0)*
	FZB24-2.5X	10.07 *(0.7)*	551 *(4.8)*	28.2 *(-3.1)*
	FZB42	9.98 *(-0.2)*	531 *(1.0)*	26.9 *(-7.5)*
	FZB42-2.5X	9.88 *(-1.2)*	530 *(0.8)*	28.3 *(-2.7)*
		(%)	*(%)*	*(%)*
Variables		*p* value		
N Rates		<0.0001	<0.0001	<0.0001
PGPR		0.89	0.92	0.72
N × PGPR		0.86	0.44	0.26

(%) relative changes in grain yield and quality traits of inoculated plants compared to the corresponding control at each N rate. All the *p* values of all PGPR treatments compared to the corresponding control at each N rate are always higher than 0.20 (not shown).

5.4 Discussion

5.4.1 Early root growth of wheat seedlings is enhanced by *B. velezensis* IT45, FZB24, and FZB42

Under gnotobiotic conditions, the three strains significantly affected the early development of both spring and winter wheat cultivars, particularly in roots. However, significant increase in shoot growth was only found in spring wheat but not in winter wheat. These contrasted responses of different genotypes of host plants to PGPR were previously described (Reynders & Vlassak 1982; Zaied et al. 2003; Araújo et al. 2013) in which it might be hypothesized that different cultivars have altered root exudate profiles that might result in different bacterial response. The strong impact of rhizobacteria on early development of wheat was also reported by (Egamberdieva 2010) with the use of pure sand as cultivation medium and a different growth duration (seven days).

5.4.2 *B. velezensis* FZB24 improves plant growth in the greenhouse but the effect is dependent on temperature and light intensity

Under greenhouse conditions, only FZB24 inoculation resulted in a significant increase in root biomass of spring wheat similar to the gnotobiotic conditions. The growth conditions in the greenhouse were approaching field conditions due to the supplemented natural light besides LED light and the influence of air temperature and microflora existing in the non-sterile soil collected from the field. Noticeably, the response of FZB24 might depend on temperature and the length of natural daytime in greenhouse. The positive result of FZB24 was obtained in May when the temperature was relatively warm (23.2 ± 2.5 °C). With lower temperature (21.9 ± 0.2 °C) and shorter length of natural daytime in October, the root growth was generally lower than that in May and no significant increase in the inoculated plants was observed. Higher temperatures and supplemented light are known to result in larger root systems in wheat (Vincent & Gregory 1989). FZB42 was also reported to increase the total root length of a spring wheat in pot experiment (Talboys et al. 2014).

5.4.3 *B. velezensis* strains enhance nutrient uptake

B. velezensis IT45, FZB24, and FZB42 are able to improve nutrient uptake of the spring wheat grown in greenhouse, but the observed increases in nutrient uptake were dependent on the analyzed organ and element. In terms of *nutrient*

concentration, the large increase in root biomass was paralleled with lowered concentrations of some macro-nutrients in the same tissues by a "*dilution*" effect. Specifically, FZB24 and IT45 increased root biomass by 31 and 17%, and that might dilute P and K concentrations in the root tissues. In contrast to macronutrients, most of the micro-nutrient concentrations were generally increased by inoculation. Hungria et al. (2010) showed lower or unchanged concentrations of some macro- and micronutrient in wheat leaves at flowering stage, but the measurement of leaf biomass increased by PGPR was not mentioned. The increase of micro-nutrient concentrations in inoculated plants were described by (Ogut & Er 2016; Singh et al. 2017). In terms of *total nutrient contents*, *B. velezensis* strains were generally able to increase both macro- and micro-nutrients contents, in which the increase in micro-nutrients was more pronounced than that of macro-nutrients. These results were consistent with other studies (Saubidet et al. 2002; Shaharoona et al. 2007; Ogut & Er 2016; Shen et al. 2016)).

Rhizobacterial IAA production has been considered as the key trait in inducing morphological root changes and increasing total root area and thus possibly leading to improved nutrient uptake by the plant (Spaepen et al. 2008; Adesemoye & Kloepper 2009; Hungria et al. 2010; Duca et al. 2014; Zahid et al. 2015; Le Mire et al. 2016). In our study, the three strains are able to produce IAA (20–36 µg mL^{-1}) in liquid culture and this agrees with previous reports (Idris et al., 2007, 2004). Bacterial knockout mutants with impaired IAA production are less efficient in promoting plant growth (Dobbelaere et al. 1999; Idris et al. 2007; Spaepen et al. 2007; Spaepen et al. 2008).

5.4.4 The effects of PGPR inoculation on winter wheat under field conditions are influenced by N rate, soil temperature, and plant stage

Only inoculation with FZB24 significantly increases the grain yield of wheat (+14.9%) in the absence of N fertilizer supply. Incidentally, this figure is quite similar to the 14.8% grain yield increase obtained by Reynders and Vlassak (1982), who also used spray application on winter wheat in Belgium but in this case *A. brasilense* strains were employed. In our 2013–2014 field trials, the absence of any N supply resulted in the highest grain yield change in inoculated plants compared to the non-inoculated control by +9.2% (mean values of the three strains). However, the higher N application rates resulted in lower grain yield changes in inoculated plants compared to the control, i.e. by +4.3% at 50N, and by +3.5% at 75N, and by +3.0% at 100N. Such N rate effect on PGPR response under field conditions were also previously reported on winter wheat (Swędrzyńska 2000; Dobbelaere et al.,

2002, with the use of *A. brasilense* strains) and on spring wheat (Reynders & Vlassak 1982; Ozturk et al. 2003; Narula et al. 2005; Shaharoona et al. 2008; Veresoglou & Menexes 2010; Milošević et al. 2012; Hussain et al. 2016; Spolaor et al. 2016). The Zeleny parameter was unaffected by inoculation with any of the three trains. Ozturk et al. (2003) also reported that grain protein of wheat was unaffected by inoculants.

Under temperate field conditions, however, the response of PGPR strains seems to correlate with air and soil temperature. The low temperature during winter might reduce the colonization and survival of PGPR when seeds are inoculated and sown in autumn. Therefore, spraying PGPR in spring time could be an alternative. In our 2014 field trial, the temperatures at the first and second inoculation were respectively 12.4 and 15.9 oC (air temperature) and 11.8 and 14.9 oC (soil temperature, 20 cm depth), while in 2015 trial they were 4.7 and 7.8 oC (air temperature) and 5.8 and 12.7 oC (soil temperature) (Figure 22). Moreover, such low air and soil temperatures in the 2015 field trial lasted during several days after inoculation, which might explain the poor performance of the strains. The optimum temperatures for the bacterial growth and the plant growth-promoting activities (such as IAA production and phosphorus solubilization) of mesophilic Bacillus species are generally higher than 20 oC (Alori et al., 2017; Mohite, 2013; Warth, 1978). Relatively warm temperature, therefore, is often recommended for PGPR application, preferable about 10 oC and more (as the product use instruction). The poor performance of PGPR under temperate field trials can also be caused by other environmental factors, such as unpredictable weather with excessive rain and deficit in sunshine (Dobbelaere et al. 2001a) or a drought (de Freitas & Germida 1992).

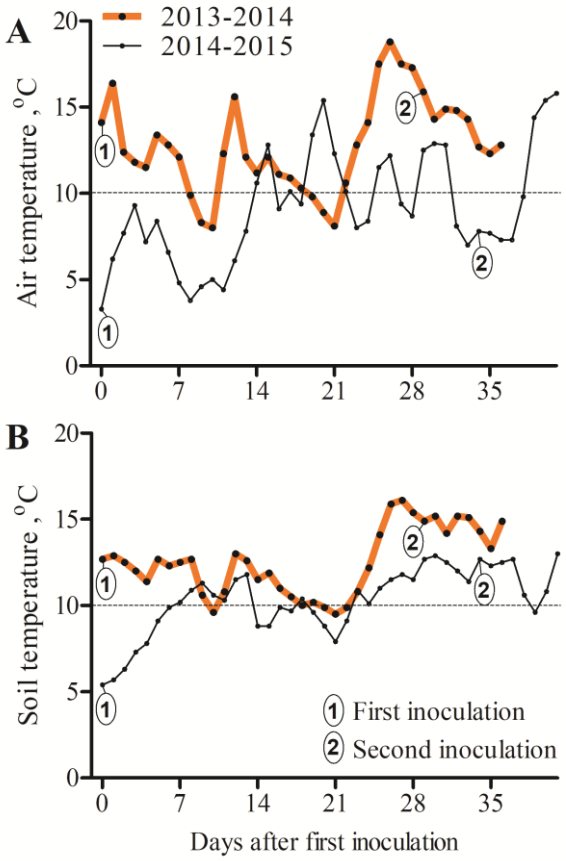


Figure 22. Air and soil temperatures in spring time when *B. velezensis* strains were applied by spraying on winter wheat in field trials in 2013–2014 and 2014–2015. (A) Daily mean of air temperature and (B) soil temperature (20 cm depth). The bacteria in both trials were inoculated a first time in spring at tillering stage (Zadok 30–31, end of March to the middle of April) and a second time at last leaf stage (Zadok 35–39, in May)

A high bacterial dose applied at the *early stages* of winter wheat in spring time seems necessary for root colonization. The spraying method at the tillering stage of winter wheat has already been successfully applied with a high density of *A. brasilense* inoculum (5×10^{10} CFU m^2) (Reynders & Vlassak 1982). In our study, the inoculation at later stage (i.e. last leaf) seems less effective than that at early stage (i.e. tillering). Specifically in the 2015 field trial, some changes supporting for

bacterial colonization took place in the last-leaf- stage inoculation, in which the soil temperature became warmer (≃12.5 ^{o}C) as compared to that of the first inoculation (tillering stage, ≃5 ^{o}C), and some of the bacterial concentrations were set up to be 2.5 folds higher than that in 2014 trial. However, such changes favorable for PGPR colonization at the last-leaf stage were not able to compensate for the negative influence of low temperatures that occurred at the tillering-stage inoculation. A higher root colonization at early stages helps PGPR to be more competitive with the often better-adapted local microflora and withstand predation by soil microfauna (Bashan et al. 2014).

5.5 Conclusions

Results obtained under greenhouse conditions are not always representative for effects of bacterial inoculation under field conditions (Veresoglou & Menexes 2010). Our work indicate evidence for consistently beneficial impacts of *B. velezensis* FZB24 inoculation on wheat growth in different cultivation systems from *in vitro*, greenhouse, to field conditions. *B. velezensis* strains are able to improve nutrient uptake in wheat, but that does not necessarily go along with higher concentrations of all nutrients in the plant tissues. Appropriate N fertilizer level, early plant stage application, and suitable temperature seem to be required to optimize the plant growth-promoting capacity of the PGPR strains on winter wheat under temperate field conditions.

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6

General Discussion, Conclusion, and Perspectives

6 General discussion, conclusion, and perspectives

6.1 General discussion and conclusion

In order to secure the global crop production while minimizing the chemical fertilizer inputs, the development of new tools able to enrich the nutrients in soil and increase the fertilizer uptake efficiency is essential (Chien et al. 2011). PGPR are therefore presented as an effective tool for such agro-ecological solutions (Calvo et al. 2014). Beneficial PGPR in rhizosphere associate with the root system and stimulate the root growth as well as support the ability to take up more water and nutrients from soil.

This study aimed at figuring out different environmental factors and farming practices which could influence the interaction between PGPR and wheat plants, such as soil properties, soil temperature, plant developmental stages, and the rate of mineral N fertilization. Besides, we aimed at assessing the relationship between nutrient uptake and the plant biomass production influenced by PGPR inoculation. There have been several previous studies dealing with this relationship, but these studies did not cover a wide range of elements, plant organs, and plant stages in the same data set (Dobbelaere et al. 2002; Hungria et al. 2010). Hence, a global analysis of nutrient uptake, including most of the common macro- and micro-nutrients in combination with different plant parts and different plant stages, was performed thoroughly in this PhD thesis.

This chapter consisted in answering the four major questions raised in the section "Thesis objectives" in Chapter 2). The answers summarize the capacities of particular PGPR strains to enhance plant growth and nutrient uptake of particular wheat varieties under particular growth conditions.

Question (1): what is the optimum N fertilization rate that would allow the PGPR strains to promote plant growth under in vitro, greenhouse, and field conditions?

Under gnotobiotic conditions, six PGPR strains –BveGB03, BmeSNji, Abr65B, BveIT45, BveFZB24, BveFZB42– are able to significantly increase root growth in the absence of any N fertilizer supplementation 14 days after sowing. Besides, three PGPR strains, BveGB03, BmeSNji, Abr65B, are able to significantly increase root growth irrespective of the N fertilization rates. The young seedling establishment phase (Zadoks 11–12) seems therefore to be independent of the N application level. This means that the plantlets rely on their own reserve in seeds and on the pre-

existing bioavailable N in the soil (Taiz & Zeiger, 2002) and the N content available in soil which might be sufficient to support their growth for two weeks.

In contrast, when the plants are growing in the greenhouse and allowed to reach older development stages (i.e. tillering and flowering stages), the plant growth-promoting activity of PGPR depends on the rate of N fertilization supply. The highest plant growth-promoting impacts of PGPR were obtained at moderate N rate, followed by maximal N, and lowest in the absence of N fertilizer supply at both tillering and flowering stages. These results are in line with two previous studies demonstrating that a moderate N rate enhances PGPR performance (Millet & Feldman 1984; Dobbelaere et al. 2002). However, there are other studies figuring out that the highest PGPR effect was observed at high N rate, followed by moderate N, and no effect at low N rate (Millet & Feldman 1984), while still other studies showed that the PGPR effect was most pronounced at low to intermediate N fertilization rates (Dobbelaere et al. 2002) or only at low N rate (Shaharoona et al. 2008; Hussain et al. 2016).

Under field conditions, the effects of PGPR inoculation on plants are also influenced by the N fertilization rate but in a different way compared to that in the greenhouse. Only one PGPR strain, BveFZB24, was able to significantly increase the grain yield of wheat in the absence of any N fertilizer supply. However, the high N application rates resulted in no significant grain yield change in inoculated plants compared to the control. Such N rate-dependent of PGPR response under field conditions were also previously reported on winter wheat (Swędrzyńska 2000; Dobbelaere et al. 2002) and on spring wheat (Reynders & Vlassak 1982; Ozturk et al. 2003; Narula et al. 2005; Shaharoona et al. 2008; Veresoglou & Menexes 2010; Milošević et al. 2012; Hussain et al. 2016; Spolaor et al. 2016).

The possible reason for such differences between greenhouse and field trials is the differences in basal N content of the soils used in those studies. The availability of N and other nutrients were limited in a small volume of soil in pot study, while the wheat growing in the fields can reach a larger volume of soil vertically and horizontally. In other words, a “moderate to high N” rate in soil in pot trials could be considered similarly to a “low N” rate in soil in field trials. Therefore, the difference in basal N content available in soil can restrict the cross-comparison of independent studies.

Question (2): Is there any relationship between the increased plant biomass caused by PGPR inoculation and the improvement of nutrient uptake in terms of nutrient concentration and total nutrient content?

In general, in term of nutrient concentration, our work shows that the plant tissues exhibiting a significantly increased biomass following PGPR inoculation will have similar to significantly lower nutrient concentrations compared to the non-inoculated controls (Figure 14). The increased biomass caused by PGPR treatments coupled with 50N correlates with lower concentrations of a few nutrients (N, P, Cu, and Mn) in root tissues of 30-day old plants (with an increase of root biomass up to +45% caused by Abr65B) and in ears of 60-day old plants (with an increase of ear biomass up to +18–23% caused by BveGB03, BmeSNji, and Abr65B). In contrast, once the rate of the increase in biomass caused by PGPR is less pronounced to insignificant, this results in similar to significantly higher nutrient concentrations as compared to the non-inoculated controls (Figure 20 and 21). For example, the increase in root biomass by only 17–31% or insignificant change in shoot biomass of plant inoculated with BveFZB42, BveFZB24, BveIT45 resulted in a reduced concentration of only two nutrients (P, K) in the root tissues, while the concentrations of other nutrients (i.e, N, Fe, Mn, Zn Cu) in these organs were increased. We hypothesized that this observation might be explained by some '*dilution*' effect, when the biomass increase caused PGPR inoculation exceeds the enhancement of nutrient uptake. In other words, the rate of the increase in plant biomass caused by PGPR inoculation and its nutrient concentrations probably have an inverse relationship. There has been few previous studies partly mentioning about this '*dilution*' effect (Dobbelaere et al. 2002; Hungria et al. 2010), but the relationship between increased biomass stimulated by PGPR and the status of nutrient concentrations were not figured out clearly.

In contrast to nutrient concentration, the total nutrient content was generally increased when there is an increase in plant biomass stimulated by PGPR. This is in agreement with other studies, in which the increase in total content of micro-nutrients was more pronounced than that of macro-nutrients (Saubidet et al. 2002; Adesemoye et al. 2010; Ogut & Er 2016; Singh et al. 2017). Several PGPR are able to solubilize complex micronutrient (i.g., Fe, Zn, Cu) compounds into simpler ones and therefore making them available to the plants (Freitas et al., 2015; Kamran et al., 2017). This ability is based on various mechanisms: (ii) acidification in the rhizosphere, and (ii) chelating micronutrients by siderophores production or proton, oxido-reductive systems on the membranes of bacterial cells or chelated ligands (Curie et al., 2013; Sharma et al., 2003; Naz et al., 2016).

Question (3): Which developmental stages of wheat are most responsive to biostimulant effects of PGPR?

Regarding plant stages, the biostimulant effects of PGPR on plant growth can be clearly observed in roots during early plant development under gnotobiotic conditions 14 days after sowing. All of the six PGPR strains (i.e., BveGB03, BmeSNji, Abr65B, BveFZB42, BveFZB24, BveIT45), which were selected from preliminary screenings under greenhouse conditions based on their plant biomass promoting capacity, are able to significantly increase root biomass. In contract, regarding shoot biomass, the promoting capacity of PGPR on shoot biomass depends on the strains and the genotypes of spring and winter wheats. Noticeably, these positive effects on biomass production caused by PGPR were independent of N fertilization.

Under greenhouse conditions, the young stages (tillering and stem elongation) of wheat after 30 days growing are generally more responsive to biostimulant effects of PGPR than later stages (after 60 days growing and reaching flowering stage). Thirty days after inoculation, the most pronounced biomass increase caused by the PGPR compared to the non-inoculated controls are up to +45% (in roots), while after 60 days inoculation the maximum increase was only up to +23% (in ears).

Under field conditions, the PGPR inoculation at later plant stage (i.e. last leaf) seems less effective than that at early plant stage (i.e. tillering) likely due to the influence of numerous environmental factors, such as soil properties, temperature and humidity, microbial community. Among those factors, the soil temperature was determined as a factor negatively influencing the PGPR application in our field trials. The strain BveFZB24 was able to significantly increase grain yield after two inoculations with warm soil temperatures (once at tillering stage with 11.8 ^{o}C and once at last leaf stage with 14.9 ^{o}C). Unfortunately, the soil temperatures in 2015 trial were lower (5.8 ^{o}C at first inoculation and 12.7 ^{o}C at second inoculation). The low soil temperature at early plant stage (i.e. tillering) seems to negatively impact the effectiveness of BveFZB24. The positive results with BveFZB24 as in 2014 field trials could not be reproduced. Although the soil temperature became warmer ($\simeq$12.5 ^{o}C) in the last-leaf stage inoculation of 2015 field trials and the bacterial concentrations of FZB24 were set up to be 2.5 folds higher than that in 2014 trial, these changes were not able to compensate for the negative impact of too-low soil temperatures occurring at tillering inoculation.

The possible explanation for the higher responses to PGPR inoculation at the early vegetative stages rather than flowering stages could be due to the secretion of

root exudates in a plant developmental stage-specific way. The secretion levels of root exudates comprising sugars and sugar alcohols, which are the main food sources of energy for the development of PGPR (Behera & Wagner 1974), were higher in early development and vegetative stages, then steadily decrease throughout the development until flowering and riping stages (Chaparro et al. 2013; Kawasaki et al. 2016). That could explain the insignificant results of 2015 field trials, during which a low-temperature period occurred early at tillering stage. A higher root colonization at early plant stages helps PGPR occupy most of the niches in a root system and/or rhizosphere, and be more competitive with other microflora (Bashan et al. 2014).

Question (4): Is the plant growth-promoting effect of PGPR reproducible in different cultivation systems, i.e. from gnotobiotic, greenhouse towards field conditions?

In our study, the possibility to observe biostimulant effects of PGPR on plant growth is generally more pronounced under well-controlled conditions, and lower under greenhouse condition and the lowest under field conditions. All of the six PGPR strains – BveGB03, BmeSNji, Abr65B, BveIT45, BveFZB24, BveFZB42 – have significant impact on plant growth under gnotobiotic conditions. However, there were only four strains (i.e., BveGB03, BmeSNji, Abr65B, and BveFZB24) that had significant impact on plant growth under greenhouse conditions. Similarly, among the three strains BveIT45, BveFZB24, and BveFZB42 which have significant impact on plant growth under gnotobiotic conditions, only one strain BveFZB24 had significantly impacted plant growth under greenhouse conditions and under field conditions.

The positive results obtained under well-controlled conditions are not considered to be representative of those obtained under greenhouse conditions, or under field conditions (Veresoglou & Menexes 2010). This is mainly due to the lack of complex interactions of PGPR with soil microbial communities and a myriad of abiotic factors (i.e., variable soil and air temperatures, humidity, air exchange, soil properties, and light intensity) under gnotobiotic conditions, while these abiotic and biotic factors always exist under greenhouse conditions and are even more prevalent under field conditions. Last but not least, our work indicates evidence for reproducible results of BveFZB24 inoculation on wheat growth from sterile (*in vitro*) conditions towards greenhouse and fully realistic conditions of field trials.

6.2 Perspectives

Improving crop productivity is still a priority to feed a fast-growing human population, and this must go along with eco-friendly agriculture, particularly in reducing the use of mineral fertilizers. PGPR have been proposed as a promising tool to enhance the chemical fertilizer uptake (Dobbelaere et al. 2002; Adesemoye & Kloepper 2009; Le Mire et al. 2016). However, this required the selection of effective PGPR which are able to increase crop productivity by coupling it with an optimum fertilizer application scheme. Our work contributed to the implementation of PGPR to increase root growth, nutrient uptake, and fertilizer use efficiency in wheat grown in temperate Europe.

This study also figures out the dependence of biostimulant effects of PGPR on N fertilization rates and also on the specific cultivation conditions, i.e., *in vitro*, greenhouse and field. Under greenhouse conditions, we showed that the highest plant growth-promoting capacity of PGPR can be observed with moderate to high rates of N fertilizers. However, under field conditions, low N fertilization seems to be necessary to improve the efficiency of PGPR. This work also determines various factors which influence the success of PGPR inoculation under different cultivationsystems, including the appropriate selection of N fertilization rate, soil substrate, plant stage application, and soil temperature. However, additional experiments are needed in order to better understand the mode-of-action, the key factors which influence the success of the PGPR inoculation under field conditions. Besides, a few specific questions remain to be addressed:

6.2.1 Interpreting the mode-of-action of PGPR under real soil condition

Understanding and quantifying the mode-of-action of PGPR under contrasted cultivation systems remain challenging. As mentioned in the section on “mode of action” in Chapter 1, PGPR are able to stimulate the plant growth by (i) several mechanisms, which stimulate plant growth and the enhancement of nutrient uptake, which involve the mechanism of production of phytohormones (e.g., auxin, cytokinins, gibberellins), volatile organic compounds (VOCs), and reducing ethylene production by ACC deaminationa, solubilization of insoluble nutrients, N_2 fixation (Bhattacharyya et al., 2012, 2014, Vacheron et al. 2013), and (ii) other mechanisms including the induction of induced systemic resistance (ISR), the synthesis of extracellular enzymes against the fungal pathogen, production of antibiotic and siderophores for iron chelation, or competition for niches in rhizosphere (Henry et al., 2013; P. Bhattacharyya & Jha 2012; Duca et al. 2014).

Such mechanisms are extensively studied under *in vitro*, well-controlled conditions or performed in liquid media. However, such conditions are too different from the conditions of a realistic soil or a cultivation system with open-air greenhouse or field trials. The complex interactions of PGPR with the environmental factors, such as soil properties and climatic factors (temperature, humidity), microflora community and plant genotypes, lead to a great challenge for the study of the mode-of-action of PGPR with the use of real soil, non-aseptic conditions or under field conditions (Calvo et al. 2014). All of the six PGPR strains used in this thesis are able to increase root growth under gnotobiotic conditions using sterile soil, but only four of them show positive results in promoting plant growth and nutrient uptake under greenhouse conditions using non-sterile soil. Therefore, studying the mode-of-actions of PGPR to explain such positive results are necessary in future studies.

Nitrogen fixation does not seem to be the main mechanism in promoting plant growth in our study. Specifically in chapter 4 (Fig. 12 and 13), the lack of biomass incease at the absence of any N supply but significant increase in biomass with additional N at 50N and 100N, at which N2-fixation has proved to be inhibited (Hartmann et al. 1986), suggested that the PGPR increased plant growth and N uptake not due to N_2-fixation. This is in agreement with Adesemoye et al. (2010) who used ^{15}N isotope approach to prove that the increased N content in plant tissues derived from the applied N fertilizer.

Rhizobacterial IAA production has been quantitatively considered as the key trait of PGPR influencing the morphological root changes among several plant-growth promoting substances produced by PGPR (Spaepen et al. 2008; Duca et al. 2014). By using a mutant of an *Azospirillum* strain producing significantly lower IAA concentration with only 10% of the wildtype *Azospirillum* strains, rhizobacterial IAA production of PGPR had been demonstrated to play a main role in root morphological and physiological changes in wheat plants (Dobbelaere et al. 1999; Spaepen et al. 2007). Hence, rhizobcaterial IAA production capacity has been selected as the first trait for screening PGPR (Etesami et al. 2015). The PGPR strains producing IAA are able to change morphology, increase total area and biomass in inoculated plants, which resulted in increase of nutrient uptake from soil (Hungria et al. 2010; Zahid et al. 2015). Therefore, the IAA production of PGPR was assayed in this thesis and it was performed in liquid culture media. All six PGPR strains (Table 5 and 6) were able to produce IAA and that could result in enhanced root biomass of plants under gnotobiotic and greenhouse conditions in our study. However, it is necessary to develop new protocols and new techniques to measure the IAA

production by PGPR under real-soil conditions (or at least root exudates should be used as growth medium for PGPR) to explain the positive results of inoculated plants.

In addition, rhizobacterial IAA could act as a reciprocal signaling molecule by influencing biological activities of other rhizobacteria. For example, co-inoculation of IAA-producing bacteria *Azospirillum* with *Rhizobium* can improve root and nodule organogenesis but also nitrogenase activity in legume plants by altering the phytohormonal homeostasis (Dardanelli et al. 2008; Remans at al. 2008). Besides, the changing in level of IAA biosynthesis regulated by the IPyA decarboxylase (IPDC) gene in *A. brasilense* can significantly alter the rhizosphere microbiota (Baudoin et al. 2010).

In our study, the IAA production capacity of PGPR in liquid culture in this thesis could be precisely quantified by high pressure liquid chromatography (HPLC) analysis or by gas chromatography-mass spectrometry (GC-MS) (Prinsen et al. 2000). The Salkowski colorimetric technique (Sarwar et al. 1992) used in this thesis is less time-consuming than HPLC and GCMS. However, it reacted not only with indole-3-acetic acid (IAA) but also with indolebutyric acid, indolepyruvic acid, and indoleacetamide (Glickmann & Dessaux 1995; Szkop et al. 2012; Goswami et al. 2015). Therefore, the quatification of IAA production by PGPR using HPLC or GCMS is necessary and less prone to artefact.

The cross-talking between rhizobacterial IAA with other phytohormones – cytokinins, gibberellins, ethylene, VOCS– produced by PGPR which results in their plant growth-promoting capacity could be further studied (as described in section 1.1.2.2: Rhizobacterial IAA and cross-talking of hormonal pathways, in Chapter 1: Bibliographical Introduction). Several mutants which are insensitive, transport/receptor deficient in hormonal signaling of auxin (*aux1*), ethylene (*ein1, ein2, eir1, etr1, ers1*), cytokinin (*ckx, cre1, ipt*), gibberellins (*gai2*), PINs (*pin)*, PLS (*pls*), double/triple mutants (e.g., s*pls-etr1, pin3-pin4-pin7*) could be used to study such cross-talking between rhizobacterial IAA with other phytohormones (Liu et al., 2017).

The auxin signalling pathway involved in PGPR response can be elicited independently from IAA production of PGPR. Zhang et al. (2007) demonstrated that volatile organic compounds (VOCs) produced by *B. amyloliquefaciens/velezensis* GB03 were able to trigger growth promotion in Arabidopsis by regulating auxin homeostasis in host plants. The GB03 strain have also been shown to have plant growth promotion effect on a model grass *Brachypodium* distachyon grown in vitro (Delaplace et al. 2015), and on wheat grown in vermiculite–soil mix in pots (Zhang

et al. 2014) watered with Hoagland solution regardless of different N rates as in our study. Although *B. megaterium* SNji produced low IAA amount in liquid culture, it was still able to significantly enhance roots and shoot biomass in gnotobiotic test (Fig.9) or pots study (Fig. 12 C, D). It was hypothesized that its low IAA production in liquid culture might not reflect precisely its realistic IAA production in rhizosphere soil, or VOCs and other mechanism could be involved in this complicated interactions (Bhattacharyya and Jha 2012). PGPRs are able to induce the root morphological changes via auxin signalling pathway by IAA and/or VOCs production and increase total root areas, thus increasing the nutrient uptake from soil and finally become a promising tool to improve plant productivity (Hungria et al. 2010; Zahid et al. 2015).

6.2.2 Could the positive results of the two best PGPR (BmeSNji and Abr65B) under greenhouse condition be reproduced under field conditions?

Three PGPR strains – *Bacillus velezensis* GB03 (BveGB03), *B. megaterium* SNji (BmeSNji), and *Azospirillum brasilense* 65B (Abr65B) – need to be further tested on spring wheat under field conditions with different N fertilization rates. These three PGPR strains apparently proved their capacity to improve plant growth of spring wheat under gnotobiotic and greenhouse conditions, and also improve nutrient uptake efficiency in several specific nutrients. The highest plant growth-promoting capacity of these PGPR strains can be obtained with moderate to high rates of N fertilizers under greenhouse conditions. Therefore, the field trials are needed to confirm whether their potential in improving wheat productivity still go along with moderate to high rates of N fertilizers as they did in greenhouse.

In order to evaluate these PGPR performance under field conditions, further experiments are firstly needed, including the optimization of the culture media and conditions for scaling-up the PGPR fermentation in bio-reactor, select the carriers and formulation, and improve the survival of the PGPR in the inoculant. These steps are critical before applying them under field conditions.

6.2.3 The optimization of inoculation methods according to current farming practices

It is necessary to continue the optimization for the implementation of the PGPR strains (BveGB03, BmeSNji, Abr65B, BveIT45, BveFZB24, BveFZB42) under field conditions to find the optimized inoculation methods fitting with the farming practices. The further optimization tests could focus on:

(1) *Inoculation methods*: seed inoculation vs. spraying at vegetative stages. The colonization of roots by PGPR can be significantly influenced by the inoculation methods and the inoculum density. *Seed inoculation* (or seed coating) method must be included in next field trials, because it is the most popular and practical application technique which is easily used by the growers and saves more labors than spraying in large farms. For instance, the seed inoculation in which the seedlings of rice were dipped in a PGPR solution resulted in an increased plant growth and such PGPR inoculation method is easier for the transplanted crops like rice (Choudhury and Kennedy, 2004). *Spraying (or drenching) inoculation* methods used under field conditions always need a larger amount of bacteria as well as special spraying/drenching equipments, as compared to seed inoculation, to be introduced to the soil in order to compete with local microbes already established in the rhizosphere (Bashan et al. 2014).

In case of crops with lower economic values, PGPR application with larger amount of bacteria under the field conditions is often costly with respect to the growers' budget. Therefore, spraying (or drenching) incoculation methods can be easily used for small volumes of soil (i.g., transplanted or nursery plants grown in pots) or drip irrigation system. Accordingly, it is suggested that PGPR applications are most promising in horticultural systems and cultivation systems conducted under well-controlled conditions of greenhouse. Such well-controlled conditions could minimize the negative impacts of abiotic and biotic stress factors occurring during the establishment phase of PGPR inoculants.

(2) *How many applications of PGPR inoculants at vegetative stages are needed to maintain the PGPR population in soil?* This is important to maximize the PGPR effects on plant growth stimulation, expecially for the inoculations at three important stages prior to the flowering stage: (i) germination, (ii) tillering and (iii) stem elongation. The higher plant growth responses to PGPR inoculation at the vegetative stages due to their higher secretion of root exudates at these stages were discussed in the answer for question 4 above (in General discussion section). The inoculation at flowering stage will be unnecessary due to its limited impacts on plant growth and seed filling. Moreover, too many times of PGPR applications with large amount of bacteria are definitely costly.

(3) *Evaluate the PGPR with different varieties of spring and winter wheat*. The performance of PGPR has been proven to be dependent on the root exudate compostitions which are determined by plant genotypes. In other words, a PGPR which does not stimulate the growth of a crop variety could well stimulate the growth of another one. Therefore, some of the PGPR strains which did not result in a

significant increase in plant growth of the wheat varieties used in this thesis (spring wheat *T. aestivum* cv. Tybalt and winter wheat *T. aestivum* L. cv. Forum) may have positive impact in other wheat varieties and this has to be confirmed in future studies.

(4) *Choosing the proper N application rates in greenhouse and field trials*. A moderate N rate for pot trials and a low N rate for field trials could be the optimum N fertilization rates for further evaluation of PGPR performance in next studies. Under field conditions, a range from absence to low N fertilizer supply (i.g, 0–25% N, or up to a maximum rate of 50% N) could be helpful and sufficient for evaluating PGPR performance. The investigation in PGPR application using high N rates from 50% up to 100% seems unnecessary. This could help to reduce a large number of replicates (plots) using for the next field trials.

(5) *Various types of N fertilizers* including mineral N fertilizers (ammonium nitrate, sodium nitrate, ammonium phosphate, urea…) or organic N fertlizers (animal-based, plant-based, manure-based..) could be used in future studies to determine the compatibility of particular PGPR inoculant with a particular type of N fertilizer, as indicated in several previous works (Kant et. al., 2010; Saia et al., 2015).

6.2.4 The strategy in the PGPR selection: native strains and consortia

(1) *Native PGPR strains*: isolating native PGPR strains from the same region site of field trials is essential to improve the survival of PGPR through winter for winter crops and enhance their competivity with local microbial community which is certainly better adapted to that local environment. There is no PGPR inoculant able to perform their best in any locations or any types of climate conditions. The local PGPR strains are able to better maintain their populations by adapting to local climate, soil properties, by being more competitive with the local microflora and withstanding predation by soil microfauna (Bashan et al. 2014). Accordingly, in order to develop a PGPR product for a particular crop, it is critical to isolate PGPR strains from the rhizosphere soil of that targeted crop. Several studies using native PGPR strains presented their positive effects on plant growth and nutrient uptake in wheat under field conditions (Swędrzyńska 2000; Mäder et al., 2011; Hassan et al., 2015; Ogut et al., 2016).

(2) *PGPR consortia*: the combination of different types of PGPR strains, or of PGPR strains with other plant growth promoting fungi, such as *Arbuscular mycorrhiza* fungi (AMF), has risen as a new trend in microbial biostimulant production, based on the promotion of both plant growth and protection. Compared to single strain application, consortia can cover most of the empty niches along the

root system, because of their increased genetic diversity and they are able to colonize most of the root zones much faster than single strains (Reddy, 2014). Such increased colonization could improve the root growth as well as nutrient uptake and/or suppress potential pathogens in the same niches. Such advantages of PGPR consortia are necessary at the beginning of the root development or seed germination prior to the colonization of other neutral or pathogenic microbes.

There are several previous studies confirming the positive results of using microbial consortia. For example, a consortia of PGPR (*B. subtilis*, *B. megaterium*, or *A. brasilense)* can result in a 33% yield increase, whereas a single strain only increases yield by about 19-24% (Turan et al., 2012). Similarly, combined strains of *B. megaterium*, *Bacillus OSU-142*, *Azospirillum brasilense* behave better than single strain in increasing plant biomass and grain yield in both greenhouse and field tests (Çakmakçı et al. 2014). Mäder et al. (2011) demonstrated that a consortia of *Pseudomonas jessenii* (R62) and *P. synxantha* (R81) with a local *Arbuscular mycorrhiza* can increase wheat yield by 41%, while PGPR strains or AMF alone increase yield by only 29% and 31%, respectively. Some PGPR strains are able to stimulate the mycorrhiza development in roots (Bianciotto and Bonfante, 2002; Cely et al., 2016). Noticeably, the compatibility in the population between the PGPR strains and/or AMF needs to be assessed before developing the formulated consortia (Bashan et al. 2014). In our study, all of the six PGPR strains are able to promote plant growth and nutrient uptake. Therefore, these PGPR strains could be combined together with PGPF to exploit their full potential in future studies.

6.3 References

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7

Scientific communications

7 Scientific communications

The following scientific communications were published during the PhD thesis:

7.1 Publications

Minh Luan Nguyen, Johann Glaes, Stijn Spaepen, Bodson Bernard, Patrick du Jardin, & Pierre Delaplace (2018) Biostimulant effects of *Bacillus* strains on wheat from *in vitro* towards field conditions are modulated by nitrogen supply. Plant Nutrition and Soil Science (accepted with minor revision).

Minh Luan Nguyen, Stijn Spaepen, Patrick du Jardin, & Pierre Delaplace (2018) Biostimulant effects of rhizobacteria on wheat growth and nutrient uptake depend on nitrogen application and plant development. Archives of Agronomy and Soil Science (in press).

Geraldine Le Mire*, **Minh Luan Nguyen***, Bérénice Fassotte, Patrick du Jardin, F. Verheggen, Pierre Delaplace, M. H. Jijakli (2016) Review: implementing plant biostimulants and biocontrol strategies in the agroecological management of cultivated ecosystems. Biotechnologie Agronomie Société et Environnment 20(S). **Co-first author*

7.2 Oral presentation

Impacts of biostimulants on promoting wheat productivity and its rhizomicrobial communities (2013). TERRA-AgricultureIsLife Seminar in Gembloux Agro-Bio Tech, University of Liège.

7.3 Posters

Minh Luan Nguyen, Bernard Bodson, Gilles Colinet, Haïssam Jijakli, Marc Ongena, Micheline Vandenbol, Patrick du Jardin, Stijn Spaepen, & Pierre Delaplace (2015) Impacts of Plant Growth-Promoting Rhizobacteria-Based Biostimulant on Wheat Growth under Greenhouse and Field Conditions. World Congress on the use of Biostimulants in Agriculture in Florence, Italy (16-19 November 2015)

Minh Luan Nguyen, Bernard Bodson, Gilles Colinet, Haïssam Jijakli, Marc Ongena, Micheline Vandenbol, Patrick du Jardin, Stijn Spaepen, & Pierre Delaplace (2015) Impacts of Plant Growth-Promoting Rhizobacteria on Wheat Growth under Greenhouse and Field Conditions. International congress: "Microbe-assisted crop production – opportunities, challenges and needs" (miCROPe 2015) in Vienna, Austria (16-19 November 2015)

Minh Luan Nguyen, Bernard Bodson, Gilles Colinet, Haïssam Jijakli, Marc Ongena, Micheline Vandenbol, Patrick du Jardin, Stijn Spaepen, & Pierre Delaplace (2014) Screening biostimulants to promote wheat productivity and its rhizomicrobial communities. 19th National Symposium on Applied Biological Sciences (7 February 2014), Gembloux Agrobiotech. Joined with a poster

Minh Luan Nguyen, Bernard Bodson, Gilles Colinet, Haïssam Jijakli, Marc Ongena, Micheline Vandenbol, Patrick du Jardin, Stijn Spaepen, & Pierre Delaplace (2014) Impacts of biostimulants on promoting wheat productivity and its rhizomicrobial communities. International Symposium on Microbial Ecology (ISME15) in Seoul, Korea (24 – 29 August, 2014)

Minh Luan Nguyen, Bernard Bodson, Gilles Colinet, Haïssam Jijakli, Marc Ongena, Micheline Vandenbol, Patrick du Jardin, Stijn Spaepen, & Pierre Delaplace (2015) Impacts of Plant Growth-Promoting Rhizobacteria on Wheat Growth under Greenhouse and Field Conditions. 10th International PGPR Workshop in Liège, Belgium (16-19 June 2015)