

# Studies on plant-microbe interaction to improve stress tolerance in plants for sustainable agriculture

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Cover: *Bacillus amyloliquefaciens* UCMB5113 colonizing *Arabidopsis thaliana* roots.

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## Abstract

Biotic and abiotic stress factors have a major impact on plants and cause extensive losses to crop production. Bacteria that provide growth promotion and prime stress tolerance of plants have great potential to improve crop production and support durable and environmental friendly resource management. Priming refers here to when plants upon appropriate stimulation develop an enhanced capacity to express defense responses to a later stimulus.

In this study strains of the beneficial bacterium *Bacillus amyloliquefaciens* were analysed for their effects on plants. Direct antagonistic effect of *B. amyloliquefaciens* on several *Brassica* phytopathogens (*Botrytis cinerea*, *Alternaria brassicae*, *Alternaria brassicicola*, *Verticillium longisporum*, and *Sclerotinia sclerotiorum*) was demonstrated by bacteria and exudates *in vitro*. A bacterial exudate fraction containing lipopeptide antibiotics was analysed and the strongest antagonistic activity was connected with a novel linear form of fengycin identified using mass spectrometry.

Growth promotion of *Arabidopsis thaliana* Col-0, *coil-16*, *jar1* and *npr1* but not in *myb72* plants by *B. amyloliquefaciens* UCMB5113 was demonstrated with increased shoot and root biomass and increased number of lateral roots and root hairs while the primary root growth decreased. *Bacillus* inoculation resulted in profound effects on various plant hormones that will affect a variety of plant functions. Growth promotion was also demonstrated in split dish experiments where *Bacillus* strains were sequestered from *Arabidopsis* plants indicating a role for volatile organic compounds (VOCs). *Bacillus* VOCs also caused growth suppression of several phytopathogens. GC-MS analysis identified a large number of compounds in the VOC blend and the composition of VOCs was dependent on the medium used for cultivation and the effects on the plant also varied.

Thus these *Bacillus* strains promote growth of plants and improve the survivability of plants exposed to biotic stress challenges by priming of stress tolerance. These findings can be extended to different crops to improve crop productivity under various environmental conditions.

*Keywords:* biotic stress factors, *Bacillus amyloliquefaciens*, *Arabidopsis thaliana*, volatile organic compounds, priming, phytopathogens.

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# Dedication

To my daughter.

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I **Shashidar Asari**, Delphine Debois, Edwin De Pauw, Marc Ongena, Sarosh Bejai, and Johan Meijer. Analysis of *Bacillus amyloliquefaciens* UCMB5113 exudates indicates multiple biological activities and support for plant protection against *Brassica* phytopathogens. (Manuscript submitted).
- II **Shashidar Asari**, Danuše Tarkowská, Jakub Rolčík, David Velázquez Palmero, Sarosh Bejai, and Johan Meijer. Analysis of plant growth promoting properties of *Bacillus amyloliquefaciens* UCMB5113 using *Arabidopsis thaliana* as host plant. (Manuscript submitted).
- III **Shashidar Asari**, Mikael Agerlin Petersen, Staffan Matzén, Sarosh Bejai, and Johan Meijer. Multiple effects of *Bacillus amyloliquefaciens* volatile compounds: growth promotion in *Arabidopsis thaliana* and growth inhibition of *Brassica* phytopathogens. (Manuscript submitted).

The contribution of Shashidar Asari to the papers included in this thesis was as follows:

- I Shashidar Asari highly involved in planning the work and performed all laboratory work except for MS analysis carried out by Ongena and co-workers. Wrote the first draft of the paper.
- II Shashidar Asari highly involved in planning the work and performed most laboratory work except for plant hormone analysis. Wrote the first draft of the paper.
- III Shashidar Asari highly involved in planning the work and performed most laboratory work except for image analysis and GC/MS analysis. Wrote the first draft of the paper.



## Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylate
AHL	N-acyl-homoserine lactone
Avr	Avirulence
BCA	Biological control agent
BR	Brassinosteroid
CK	Cytokinin
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
GA	Gibberellin
GC	Gas chromatography
GMO	Genetically modified organism
HPLC	High performance liquid chromatography
HR	Hypersensitive response
IAA	Indole acetic acid
IR	Induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
LBA	Luria broth agar
M9A	Minimal medium agar
MAMP	Microbe associated molecular pattern
MSA	Murashige and Skoog agar
NB-LRR	Nucleotide binding-leucine rich repeat
NRPS	Nonribosomal peptide synthetase
PAMP	Pathogen associated molecular pattern

PDA	Potato dextrose agar
PR	Pathogenesis related
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
SA	Salicylic acid
SAR	Systemic acquired resistance
TSA	Tryptic soy agar
VOC	Volatile organic compound

# 1 Introduction

Agriculture is essential to provide mankind with an adequate basic source of food supply. The rapid increase of the world population and the reduction in arable land available for crop cultivation as a consequence of urbanization are rising challenges to future food security (Nellemann *et al.*, 2009). Increasing crop losses may also be expected due to increased stress factors (biotic - e.g. pathogens and insects, and abiotic - e.g. drought, heat and cold) due to changes in trade, agricultural practices, climate and other factors. The current main approach for plant protection from pathogens and insect pests is based on the use of chemical pesticides, but these cause environmental pollution and health hazards to humans beyond their intended use. An increase in agricultural productivity is needed for the future and that should be based on sustainable practices that minimize the environmental impact but at the same time support food safety and food security.

## 1.1 *Brassica* crops

The family *Brassicaceae* contains many plants important for food and fodder production. Common commercial *Brassica* crops are oilseed rape, mustard, cauliflower, cabbages, broccoli, Brussels sprouts, collard greens, kale, radish and turnip. Oilseed rape (*Brassica napus*) is an important source of vegetable oil and presently the major oil crop within the EU and the third worldwide next to soybean and sunflower. The seed oil is rich in omega-6/omega-3 fatty acids and the vegetative parts contain secondary metabolites known as glucosinolates. The enzyme myrosinase hydrolyzes glucosinolates into isothiocyanates, thiocyanates, nitriles and other toxic products to defend plants under attack but these products also serve in biofumigation (Ahuja, 2010; Szczyglowska *et al.*, 2011; Hu *et al.*, 2015). The production of oilseed rape is increasing because of its nutritional and health benefits. Glucosinolate products can prevent cancer and have other health promoting effects (Talalay

and Fahey, 2001; Kapusta-Duch *et al.*, 2012). In addition to being used for human consumption, oilseed rape is also used in fodder and production of biodiesel.

## 1.2 Stress factors on plants (biotic and abiotic)

Plant exposure to both biotic and abiotic stress factors cause major losses to crop production worldwide (Boyer, 1982; Bray *et al.*, 2000; Suzuki *et al.*, 2014). Biotic stress factors, resulting from interaction with other organisms, include infection by pathogens or damage by insect pests. Abiotic stress factors include extreme temperature, drought, water logging and salinity as major parameters that affect plant growth. Different strategies are used to control stress in plants including, 1) chemical pesticides: that however create health hazards and environmental pollution and according to EU REACH/CAP programs should be reduced in coming years, 2) conventional plant breeding: which is time consuming and requires availability of resistant varieties, and 3) genetically modified organisms (GMO): that are not favoured in EU.

Alternative solutions aim to develop environment-friendly strategies using biological agents that provide biotic/abiotic stress tolerance by strengthening plants natural defense (“resistance inducers”) often based on so called priming (Conrath *et al.*, 2015).

## 1.3 Plant roots and exudates

The root is a vital organ of plants and is the first to arise as the radicle from the germinating seed. The root system consists of embryo derived primary root, post-embryonically derived secondary roots and root hairs. This root system plays a vital role in uptake of water and nutrients, mechanical support providing anchorage to the ground, and for food and nutrient storage. The root can modify its geometry according to the environment (Ditengou *et al.*, 2008; Pacheco-Villalobos *et al.*, 2012; Smith and De Smet, 2012).

Roots can continuously synthesize, accumulate and secrete a wide range of compounds into the soil (Bais *et al.*, 2001; Baetz and Martinoia, 2014). These compounds are first accumulated, then transported across the cellular membrane and excreted into the rhizosphere. The rhizosphere can be described as a narrow region of soil around the root area that is associated with soil microbial communities, mostly bacteria, that is influenced by root secretions (Haghighi *et al.*, 2011; Berendsen *et al.*, 2012). The root exudate contains H<sup>+</sup> ions, water, enzymes, mucilage and carbon containing primary and secondary compounds (Uren, 2000; Wen *et al.*, 2007; Faure *et al.*, 2009). Based on the

active processes of root exudation, the compounds are classified into two groups, the first class consists of “waste” materials with unknown function and the second class contains lubrication and defense compounds with known function (Uren, 2000; Bianciotto *et al.*, 2001; Dennis *et al.*, 2010; Baetz and Martinoia, 2014). Further, compounds in root exudates are classified based on the size as low or high molecular weight compounds. These compounds vary depending on the type of soil profile, plant age, environmental conditions and nutrient availability (Brady and Weil, 1999; Neumann *et al.*, 2014). The root exudates may act in signaling to direct positive and negative interactions between plants and microorganisms in the rhizosphere (Fig. 1).

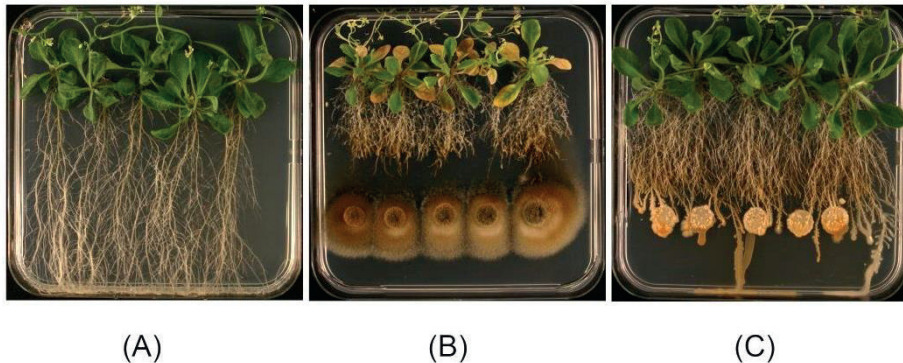


Figure 1. Some possible outcomes of plant-microbe interactions with *Arabidopsis thaliana* Col-0 plants and different microorganisms. Control (A), negative effect on plant (disease) (B), and positive effect on plant (growth promotion) (C).

## 1.4 Biocontrol agents

Biological control is defined as the reduction of pest and pathogen effects by natural enemies. Biological control agents (BCA) include predators, parasitoid insects and microorganisms (bacteria, fungi and viruses) that are used to control biotic and abiotic stress factors and are considered eco-friendly strategies to produce more healthy crops and improve crop yield. Certain beneficial microorganisms are known for their ability to enhance plant growth and serve as biopesticides (here defined as an agent based on a living microorganism or a natural product used for the control of plant pests) (Chandler *et al.*, 2011; Melo *et al.*, 2014). Many studies have demonstrated the ability of plant growth promoting rhizobacteria (PGPR), plant growth promoting bacteria (PGPB) and plant growth promoting fungi to stimulate plant growth (Hamayun *et al.*, 2010; Masunaka *et al.*, 2011; Amaresan *et al.*, 2013; Ahmed and Hasnain, 2014; Gutjahr, 2014; Kakoi *et al.*, 2014;

Pankievicz *et al.*, 2015), improve protection against biotic stress (Barriuso *et al.*, 2008; Hammami *et al.*, 2013; Kojima *et al.*, 2013; Park *et al.*, 2013; Shimizu *et al.*, 2013; Hossain *et al.*, 2014), and improve tolerance to abiotic stress (Marasco *et al.*, 2013; Caporale *et al.*, 2014; Nadeem *et al.*, 2014; Ullah and Bano, 2015; Sukweenandhi *et al.*, 2015). Therefore, since a few years researchers have been focusing on improving plant growth and yields using effective BCA to support more sustainable agriculture. This study is focused on systemic analysis of plant responses to interactions with a PGPB, *Bacillus amyloliquefaciens*. *B. amyloliquefaciens* strains are Gram-positive spore forming bacteria common in soil that may stimulate plant growth and control negative effectors.

#### 1.4.1 Plant-microbe interaction

Many studies have enabled and improved our understanding of some of the physiological processes connected with roots in the soil, chemical compounds secreted by roots, communication between microbes and root system dynamics (Fujishige *et al.*, 2006; Rudrappa *et al.*, 2008a; Rinaudi and Giordano, 2010; Pangesti *et al.*, 2013; Gutjahr, 2014; Kakoi *et al.*, 2014) and possible defense mechanisms. A number of symbiotic associations between many plants and microorganisms have evolved where special attention has been given to root associations. Mycorrhiza is distinguished by fungal colonization intracellularly or extracellularly that support nutrient acquisition (Field *et al.*, 2015). Rhizobia is established when certain bacteria form root nodules of legumes and can fix nitrogen and make it available to the plant (Gourion *et al.*, 2015). These associations have provided information about some of the barriers that exist and need to be overcome to establish a mutualistic relationship since plants have developed constitutive and inducible defense systems to avoid detrimental interactions.

#### 1.4.2 PAMPs/MAMPs

Plants fulfil particular needs to many microorganisms. Plants and microbes communicate with each other by use of different signaling molecules during their interaction. Many microbes can be harmful to plants affecting growth and survival. Plants are capable of recognizing certain compounds released by microbes and mount inducible defense. The interaction between plants and microbes leads to the activation of local and systemic defenses under control by plant signaling hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) and depend upon the nature of the microbe (Koornneef and Pieterse, 2008; Yi *et al.*, 2014). Plants recognize pathogens directly or indirectly. In direct recognition, plants can detect extracellular molecules

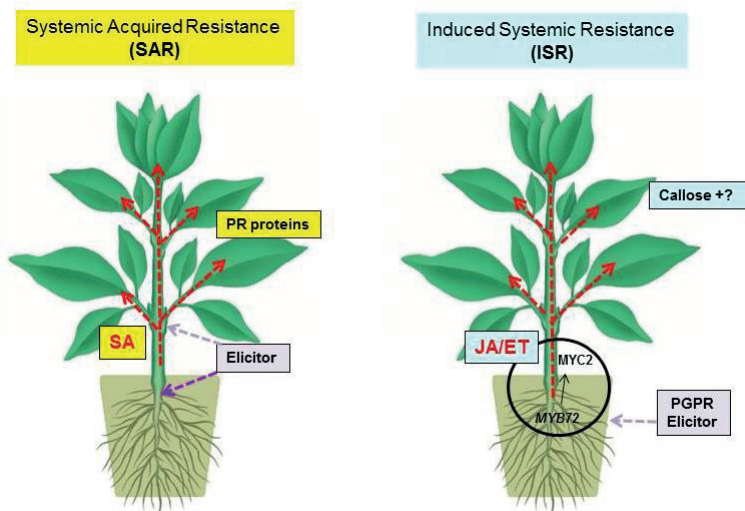
referred to as Pathogen associated molecular patterns or Microbe associated molecular patterns (PAMPs/MAMPs), e.g. bacterial flagellin, Ef-TU proteins, lipopolysaccharides and peptidoglycans (Boller and Felix, 2009), and/or intracellular effector proteins, e.g. Avr3a, AvrK and Avr10 proteins or tissue damage using pattern recognition receptor (PRR) proteins located on the cell surface or intracellularly (Allen *et al.*, 2004; Rivas and Thomas, 2005; Boller and Felix, 2009). According to Jones and Dangl (2006), the plant immune system can be represented in a four phase 'zigzag' model. In phase 1, PAMPs of microbes are recognized and activate the plant immune system by binding to specific PRRs located on the cell surface (Bakker *et al.*, 2007; Boller and Felix, 2009; Mahmut *et al.*, 2009; Newman *et al.*, 2013). PRR recognition results in PAMP-triggered immunity (PTI) that prevents colonization and proliferation. In phase 2, certain pathogens produce effectors that increase virulence. These effectors can interfere with PTI and result in effector-triggered susceptibility (ETS). In phase 3, if the effector is specifically recognized by nucleotide binding-leucine rich repeat (NB-LRR) receptor proteins, effector-triggered immunity (ETI) is activated resulting in disease resistance e.g. manifested as a hypersensitive response (HR) at the infection site. In phase 4, natural selection may have driven pathogens to suppress ETI by shedding or developing additional effectors promoting virulence until plants have developed novel receptors.

#### 1.4.3 Inducible defense (IR, SAR, ISR)

Plants are attacked by many different pathogens and insect pests. To stop infections, plants have developed an advanced immune system by combining constitutive and inducible defense responses of many different kinds (Hammerschmidt, 1999; Nürnberger *et al.*, 2004; Návarová *et al.*, 2012). Formation of reactive oxygen species and programmed cell death at the site of the infection observed in HR occurs due to the so called gene-for-gene interaction between plant resistance (R) genes and pathogen avirulence (Avr) genes.

Plants can induce resistance both locally and systemically to subsequent attack by the same or different pathogens (Walters *et al.*, 2005; Hammerschmidt, 2007). This induced resistance (IR) may control the pathogens or damaging factors, completely or partially (Kuc, 1982; Chen *et al.*, 2014). Several studies have shown that genes expressed during IR responses produce proteins with chitinase, glucanase and other enzymatic activities that are involved in defense reactions to a wide spectrum of pathogens (Van Loon *et al.*, 2006).

The activation of defense mechanism throughout the plant can be managed in different ways and two common routes are referred to as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse *et al.*, 2012). The plant hormones SA and JA are involved in systemic resistance pathways and are known to reduce the effect of pathogens on plants (Kniskern *et al.*, 2007; Makandar *et al.*, 2010). The induction of plant defense as SAR or ISR is in a simplified scheme dependent on SA or JA and ET, respectively (Pieterse *et al.*, 2012). Both types of systemic defense can be differentiated on the basis of nature of the elicitor and differences in regulatory signaling pathways demonstrated in a model plant system (Schenk *et al.*, 2000; Van Wees *et al.*, 2000; Yan *et al.*, 2002) shown in (Fig. 2) (Vallad and Goodman, 2004).



*Figure 2.* Two forms of induced defense in plants. Systemic acquired resistance (SAR), induced by exposure of below- or aboveground tissues to biotic or abiotic elicitors, dependent on salicylic acid (SA) signaling and resulting in accumulation of pathogenesis-related proteins (PR proteins). Induced systemic resistance (ISR), induced (primed) by exposure of roots to plant growth-promoting rhizobacteria (PGPR), activation of transcription factors (MYB72 and MYC2) and dependent on jasmonic acid (JA) and ethylene (ET) signaling, stimulating e.g. callose formation but generally not PR proteins. Both pathways need the NPR1 gene though.

In SAR enhanced resistance even in distal parts of the plant provide protection against the attack of the same or different pathogens (Sticher *et al.*, 1997; Jing *et al.*, 2011; Shah and Zeier, 2013). The SAR can be triggered by exposing the plants to various pathogenic and non-pathogenic microbes or chemicals such as SA or artificial chemicals including, 2,6-dichloro-isoicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Sticher *et al.*, 1997). Typical for SA mediated SAR is the induction of



pathogenesis related (PR) proteins like PR-1, PR-2 and PR-5 (Cameron *et al.*, 1994; Van Loon LC, 1997; Jones and Dangl, 2006).

Priming refers to a special state where plants will be more tolerant or resistant to stress upon challenge as a result of an earlier appropriate stimulus. PGPR colonize the root and induce systemic resistance in areal parts of the plant, which is known to protect plants from pathogens (Bais *et al.*, 2004; Lugtenberg and Kamilova, 2009). Priming of disease resistance by BCA was proposed to act through a novel signal transduction pathway resulting in ISR different to the classical SA-dependent SAR. In ISR neither PR proteins nor SA seems to be involved (Yan *et al.*, 2002). Colonization of *Arabidopsis* root by PGPR (*Pseudomonas*) was shown to result in ISR dependent on JA and ET signaling (Van Loon, 1997; Kloepper *et al.*, 2004). The MYB72 transcription factor interacting with EIL3 seems to be key players while the NPR1 gene is also needed for ISR (Van der Ent *et al.*, 2008). The other transcription factor MYC2 can also play a potential role in regulating JA gene expression during rhizobacteria-mediated ISR against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Pozo *et al.*, 2008). Priming of ISR is considered to have low fitness costs since defense is only activated at stress challenge while e.g. treatment of plants with SA or JA induce plant defense and disease resistance immediately although no stress is imposed (van Hulten *et al.*, 2006).

#### 1.4.4 Impact of PGPB on plants

Soil is a heterogeneous matrix with various dynamic parameters, varying in nutrients and organic matter (0.8 to 2%), the levels of which are boosted by the presence of plants. Hence, plant-associated microorganisms face a scarcity of nutrients during growth in soil absent of plants. Therefore, soil bacteria enter stationary phase at nutrient insufficiency and remains in this stationary phase until nutrients can be obtained (Kolter *et al.*, 1993). Plants acquire nutrients from the soil which are utilized for development. Plant root architecture plays a vital role in uptake of water, nutrients and anchorage to the soil (Flores *et al.*, 1999; López-Bucio *et al.*, 2003). Plants face continuous challenges in the rhizosphere due to soil microbiota competing for nutrients and space. In the rhizosphere the microbial community has been estimated to constitute about  $10^{10}$  bacteria per gram of soil (Gans *et al.*, 2005; Roesch *et al.*, 2007). About 5 to 21% of photosynthetically fixed carbon in the plant is transferred in the form of root exudates into the rhizosphere (Marschner, 1995; Hernández *et al.*, 2015). These root exudates are rich in small molecules that contain low (amino acids, organic acids, sugars, phenolics and secondary metabolites) and high (polysaccharides and proteins) molecular mass compounds (Walker *et al.*, 2003; Bais *et al.*, 2006; Shi *et al.*, 2011) that act as chemical signal attractants

and/or repellents towards a complex mixture of diverse microbes in the rhizosphere that includes bacteria, fungi, algae, protozoa and nematodes (Ryan

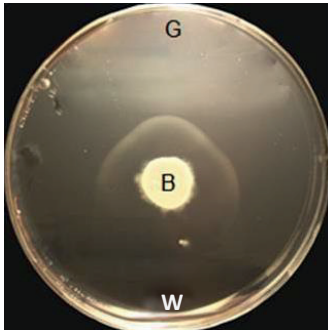


Figure 3. Chemotactic response of *Bacillus amyloliquefaciens* UCMB5113 towards glucose for their carbon source on phosphate buffer agar plate. Glucose (G), *Bacillus* (B), and water (W).

*et al.*, 2001; Badri and Vivanco, 2009; Gaiero *et al.*, 2013). Microbes find root surfaces through chemotactic responses to plant exudates. Increased concentrations of certain compounds in the rhizosphere therefore stimulate microbial colonization of the rhizoplane (Fig. 3).

This root exudation process stimulates colonization and plant growth through several direct and indirect mechanisms (Couillerot *et al.*, 2009; Richardson *et al.*, 2009) where certain nutrients are available enabling proliferation and increased microbial populations (Bais *et al.*, 2006; Pothier *et al.*, 2007; Badri *et al.*, 2009; Shukla *et al.*, 2011; Drogue *et al.*, 2013) and establishment of colonization of the plants. In return, PGPB enhance plant growth and/or antagonism towards phytopathogens resulting in mutually beneficial relationships. Interactions between organisms have been divided into three classes based on the mode of interactions and relationship; symbiotic/mutualism (e.g. BCA), commensalism or parasitism on plants.

In a mutualistic relationship: two organisms of different species interact with each other and work together where both benefit. In commensalism interaction one organism benefits from the other without harming it. In parasitism one organism is harmed while the other benefits.

Certain PGPB can also improve plant growth and tolerance against environmental stress (both biotic and abiotic). Plants are exposed to various environmental stresses and plant hormones play a crucial role in signaling including compounds such as abscisic acid (ABA), JA, SA, and ET that respond to stress protecting plants from different biotic and abiotic stresses (Fujita *et al.*, 2006). Generally plants synthesize low amounts of ET that is

beneficial for plant growth and development. However, during stress responses in plants the increased ET biosynthesis is referred to as ``stress ethylene`` (Stearns and Glick, 2003; Glick *et al.*, 2007) that is a response to biotic and abiotic stress factors (Stearns and Glick, 2003; Lim and Kim, 2013). ET stimulates abscission, chlorosis, and senescence in plants and leads to plant growth inhibition and tissue death. However, studies show that 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity of PGPB can control the stress ET concentration in plants (Hao *et al.*, 2007; Ali *et al.*, 2014; Glick, 2014).

#### 1.4.5 Bacterial mineralization

Plants cannot directly take up compounds like nitrogen, iron, and phosphate which are abundant in the soil. It is known that many members of the PGPB community associated with the plant root system can convert atmospheric nitrogen into ammonium and provide it to plants by the nitrogen fixation process (Richardson *et al.*, 2009; Chaparro *et al.*, 2013). Several studies have shown that the majority of nitrogen-fixing organisms are *Rhizobium* spp. and in agricultural practice these nitrogen fixing PGPB are routinely used to inoculate plants to increase the number of nodules and plant biomass (Ma *et al.*, 2003; Govindarajan *et al.*, 2007). Next to fixed nitrogen, phosphorous is an essential element for plant growth. Phosphorous is found in soil, mostly in an insoluble state that plants cannot use directly. Many soil bacteria convert insoluble phosphorous and phosphate to soluble forms by producing low molecular weight organic acids such as gluconic and citric acid. In the soil the amount of bioavailable iron is very low due to its accumulation as iron oxides and hydroxides that cannot be readily utilized by living organisms (Kraemer, 2004). Thus, there is a competition for iron among the organisms in the rhizosphere. Bacteria secrete siderophores which are low molecular weight chelating compounds with high-affinity for iron. The siderophore-producing PGPB facilitate plant iron acquisition from iron-limited environments and can sequester iron from neighboring microorganisms outcompeting them (Whipps, 2001; Lodewyckx *et al.*, 2002; Xianmei *et al.*, 2011; Ahmed and Holmström, 2014) as well as plant pathogens, leaving low amounts of bioavailable iron behind (Kloepper *et al.*, 1980; O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999; Fones and Preston, 2013). In addition, many PGPB and BCA have been thoroughly studied for production of antibiotics (e.g. Ezra *et al.*, 2004; Amin *et al.*, 2012; Gutiérrez-Chávez *et al.*, 2015; Inès and Dhouha G, 2015) and lytic enzymes like hydrolases (Chernin and Chet, 2002; Sawant *et al.*, 2015), chitinases (Frankowski *et al.*, 2001; Tan *et al.*, 2015), laminarinases

(Lim *et al.*, 1991; Labourel *et al.*, 2014), and glucanases (Singh *et al.*, 1999; Tan *et al.*, 2015).

#### 1.4.6 Phytohormones

Plants respond and adjust to environmental changes by altering phytohormone levels. Many studies have reported that PGPB stimulate plant growth by direct or indirect mechanisms. In direct mechanisms bacteria produce phytohormones, e.g. indole acetic acid (IAA), gibberellins (GAs), cytokinins (CKs) and ET (Tien *et al.*, 1979; Williams and Sicardi De Mallorca, 1982; Badenoch-jones *et al.*, 1984; Taller and Wong, 1989; Nieto and Frankenberger, 1989; Patten and Glick, 2002; Glick, 2012; Rajkumar *et al.*, 2013; Ahmed and Hasnain., 2014; Fierro-Coronado *et al.*, 2014; Etesami *et al.*, 2014; Khan *et al.*, 2014; Kudoyarova *et al.*, 2014) that stimulate plant growth and/or modulate the hormone level in plants that may also support antagonism to phytopathogens. In indirect mechanisms the bacteria induce plant immunity by producing molecules that can modulate the hormone level. Studies have shown that inoculation of CK or GA producing PGPB stimulated plant growth (Lorteau *et al.*, 2001; Joo *et al.*, 2009; Kang *et al.*, 2009). IAA producing PGPB enhanced plant growth in canola (Patten and Glick, 2002), tomato (Mayak *et al.*, 1999), mung beans (Xie *et al.*, 1996), rice (Bal *et al.*, 2013) and *Brassica juncea* L. (Indian mustard) (Shim *et al.*, 2015). PGPB can also stimulate plant growth by expressing the enzyme ACC deaminase that cleaves ACC to  $\alpha$ -ketobutyrate and ammonia, decreasing the ET level in plants (Penrose and Glick, 2003; Glick, 2005; Sessitsch *et al.*, 2005; Sun *et al.*, 2009). ET plays a crucial role in plant development as well as in stress signaling (Frankenberger and Arshad, 1995; Glick, 2014; Schaller, 2012). ET stimulates seed germination, root hair development, root elongation, fruit ripening, opening of flowers, and abscission of leaves. However, during stress conditions in plants the production of ET (“stress ET”) is so high that it antagonizes plant growth.

### 1.5 Root colonization

Rhizobacteria can colonize plant roots at all stages of plant development and they can multiply on roots to build a mutual relationship between plants and microorganisms, where this interaction provide benefits to both partners (Hallmann *et al.*, 1997; Reiter and Sessitsch, 2006). The microbial community structure and its ability to metabolize and compete for carbon sources in the rhizosphere are dependent on the amount and composition of plant root exudates (Klopper *et al.*, 1992; Lazarovitis and Nowak, 1997; Farrell *et al.*,

2014). Once the bacteria colonize the root they can be epiphytic and/or endophytic. Epiphytic bacteria stay and live on the surface of the roots. Endophytic bacteria can penetrate into the root and even systemically spread into the aerial parts of the plant and vascular tissue cortex, xylem and pith (Reinhold-Hurek and Hurek, 1998; James, 2000). Many studies suggest the mode of Gram-negative and Gram-positive bacteria penetration into the root through the main root, lateral roots and root hair (Estrela Borges Baldotto *et al.*, 2011; Huang *et al.*, 2011; Prieto *et al.*, 2011).

### 1.5.1 Bacterial endophytes

Endophytic bacteria colonize plant tissues without causing injuries to the host plant (Bacon and Hinton, 2006). The endophytes first colonize the rhizosphere (root surface) and then form a biofilm on the host tissue (Sturz *et al.*, 2000). Like phytopathogens, endophytes utilize specific mechanisms to enter into the plants. The endophytes may enter into the plant through different ways depending on the bacterial and plant species interacting.

### 1.5.2 Biofilms

PGPR are recognized among the plant associated soil microbial communities to enhance plant growth (Lugtenberg and Kamilova, 2009). These PGPR are effective in colonizing the plant root and further multiply into microcolonies and/or produce biofilm as a result of a successful plant-microbe interaction (Saleh-Lakha and Glick, 2006). The plant associated biofilms are highly capable of providing protection from external stress, decreasing microbial competition, and give beneficial effects to the host plant supporting growth, yield and crop quality (Ramey *et al.*, 2004). In biofilm formation processes a single microbial cell adheres to a surface (abiotic or biotic), it multiplies to form multiple microcolonies, in which the cells are linked to each other and embedded in a matrix of extracellular polymeric substances called exopolysaccharides (Fig. 4). Biofilms can also contain extracellular DNA, proteins and other compounds. The growing cells in the biofilm are distinct both phenotypically and in gene regulation compared to planktonic cells of the same organism.

To date, many *Pseudomonas* spp. and *Bacillus* spp. are reported to be able to colonize plant leaves or root surfaces and are capable of biofilm formation (Ude *et al.*, 2006). The colonization of microbes on plant roots depends upon root exudates for nutrition and carbon source (Bais *et al.*, 2006). By producing organic compounds as a carbon source, root exudates play a central role in

triggering root colonization (Lugtenberg *et al.*, 1999; Lugtenberg and Kamilova, 2009).

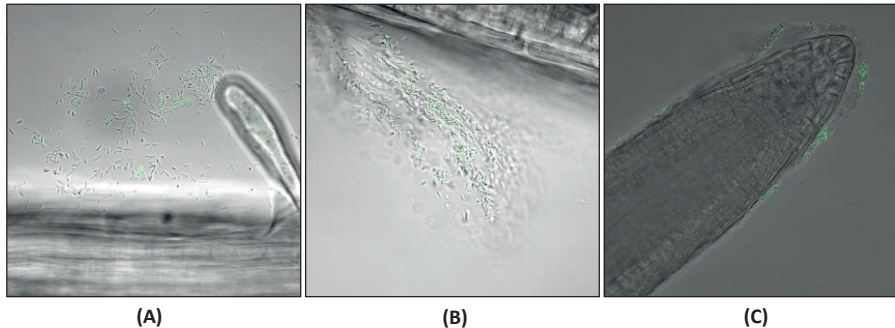


Figure 4. Colonization and biofilm formation of *B. amyloliquefaciens* UCMB5113 on roots of *A. thalaina* (A-C).

### 1.5.3 Quorum sensing

During the formation of biofilm the bacteria communicate chemically with each other by quorum sensing. This helps the microbial communities to respond quickly, inhibiting competing organisms, improving nutrient uptake, and help to adapt to changing environmental conditions. Also it controls bacterial size and population status. N-acyl-homoserine lactones (AHLs), 2-heptyl-3-hydroxy-4-quinoline and autoinducer-2 are examples of diffusible signals that are used in cell-cell communication within the bacterial community to synchronize some actions and make them function more like a single unit. These signaling molecules are unique among the microbial species. AHLs in *Proteobacteria*, gamma-butyrolactones in *Streptomyces*, cis-11-methyl-2-dodecanoic acid in *Xanthomonas* and oligopeptides in Gram positive microbes are examples of such signal molecules (Danhorn and Fuqua, 2007).

### 1.5.4 Swarming motility

Swarming motility is defined as translocation of coordinated bacterial populations across solid or semi-solid surfaces. Swarming motility is due to the formation of dendritic fractal-like patterns formed by cells migrating from an initial location and is dependent on the nutrient composition and viscosity of the culture medium (Fig. 5). Swarming motility is one of the bacterial surface translocation modes among six described forms, such as swimming, darting, gliding, twitching and sliding (Henrichsen, 1972; Jarrell and McBride, 2008; Shrout, 2015). The three steps involved in swarming motility are 1) formation

of a regular colony, 2) cell differentiation at the initiation rim point of the colony, and 3) formation of hyperflagellated swarmer cells. The fast multiplication and movement of swarming cells results in rapid surface colonization (Eberl *et al.*, 1999; Kearns, 2010). In swarming motility the bacterial cells are translocating on a surface-linked in a network with neighbouring bacteria by extensive flagella. In swarming motility the bacterial cells move rapidly on a solid or semi-solid surface in a coordinated way. Flagella are required for production of a viscous slime layer in *in vitro* conditions and maintain a moist environment (Verstraeten *et al.*, 2008). Swarming is a common ability for many PGPR such as *Bacillus* (Kearns and Losick, 2004) and *Pseudomonas* strains (Dézziel *et al.*, 2003; Tremblay *et al.*, 2007; Oura *et al.*, 2015).

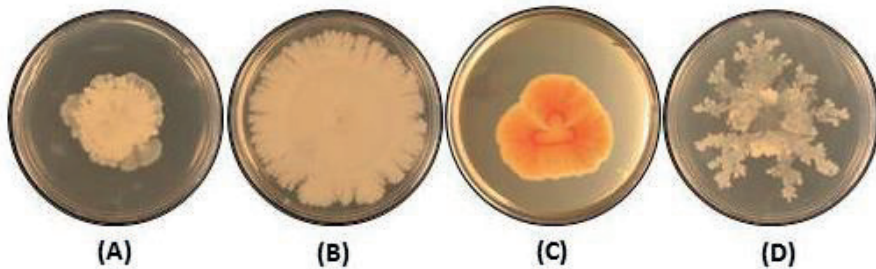


Figure 5. *B. amyloliquefaciens* strains swarming motility on PDA plates. (A) UCMB5033, (B) UCMB5036, (C) UCMB5113 and (D) FZB42.

The chemotaxis sensory system might also be involved in swarming motility which shows various swarming patterns in different species (Partridge and Harshey, 2013).

In some bacterial species, biosurfactant synthesis is required for swarming motility which is under the control of the intercellular quorum sensing communication system (Harshey and Matsuyama, 1994; Daniels *et al.*, 2004).

### 1.5.5 Lipopeptides

*Bacillus* spp. are well studied for production of a wide range of lipopeptides (LPs) (Ongena and Jacques, 2007). LPs contain a lipid tail connected to a short linear or cyclic oligopeptide and are produced by various microorganisms (Raaijmakers *et al.*, 2010). These LPs are synthesised non-ribosomally by large multi-enzyme complex nonribosomal peptide synthetases (NRPSs) and antagonise other microorganisms including phytopathogens (Stein, 2005; Finking and Marahiel, 2004). LPs includes surfactins, fengycins, iturins,

bacillomycin, bacilysin, lichenysin and mycobacillin (Ongena and Jacques, 2008; Aleti *et al.*, 2015). Another major group of secondary metabolites produced by *Bacillus* is polyketides that also can serve in antagonism (Aleti *et al.*, 2015).

## 1.6 Volatile organic compounds (VOCs)

Volatiles are organic compounds that contain a high vapour pressure at room temperature. Most of the VOCs are scents or odors, lipophilic with a small molecular mass (<300 Da) and derivatives of terpenoids, phenylpropanoids, fatty acids and various sulfur and nitrogen containing compounds. These VOCs not only diffuse into the atmosphere above ground but can also diffuse into the below ground with similar complexity. Due to this property, these compounds are essential for inter-and intra-species attraction, recognition, communication, repellent action and defense (Wenke *et al.*, 2010).

### 1.6.1 Plant volatiles

Plants emit volatile substances with distinctive smells from different tissue parts during growth and development into the atmosphere (Pichersky and Gershenzon 2002; Peñuelas and Staudt, 2010). It has been reported that plants emit more than one thousand low molecular mass organic compounds including terpenes, isoprenes, acids, alcohols, alkanes, alkenes, carbonyls, esters and ethers (Knudsen *et al.*, 1993; Kesselmeier and Staudt, 1999). The rate of production and emission of terpenes are modulated by biotic and abiotic factors (Peñuelas and Lusia, 2001; Paris *et al.*, 2010). Various environmental factors such as light and temperature influence the production of plant volatiles being higher in summer and in midday (Kesselmeier and Staudt, 1999).

The volatiles from flowers serve to attract pollinators and seed dispersers (Reinhard *et al.*, 2004). The volatiles emitted from infested plants serve in interactions and/or defense to pests, pathogens, and herbivores (Farag *et al.*, 2013). Plants release volatiles along with root exudates into the soil. The microorganisms and their population in the rhizosphere utilize these volatiles as infochemicals for diverse interactions (Wenke *et al.*, 2010) (Fig. 6).



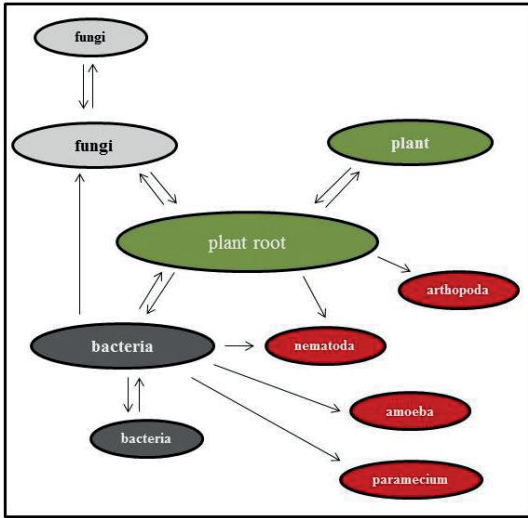


Figure 6. The scheme presents the possibility of intra-and interspecies interaction in belowground plant volatiles. The arrows show effects. (The figure from Wenke *et al.*, 2010 is reproduced by publisher permission).

### 1.6.2 PGPR volatiles

PGPR can produce a complex blend of volatiles which are distinct between bacterial species and closely related species (Groenhagen *et al.*, 2013; Garbeva *et al.*, 2014). Certain of these bacterial volatiles can stimulate plant growth (Ryu *et al.*, 2003, 2004; Zhang *et al.*, 2007; Xie *et al.*, 2009; Farag *et al.*, 2013), cause disease suppression by stimulating ISR (Rudrappa *et al.*, 2010), or antagonize phytopathogens (Kai *et al.*, 2007; Vespermann *et al.*, 2007) nematodes and insects (Kai *et al.*, 2009).



## 2 Aim of this study

Use of plant growth promoting bacteria to control both biotic and abiotic stress factors are considered as eco-friendly strategies to produce more healthy crops and increase crop yield. However, several properties need to be addressed to better understand requirements for the desired effects and then to optimize utilization for agricultural practices. The main aim of the present work was to evaluate the potential of *Bacillus amyloliquefaciens* strains to improve growth and stress tolerance in *Arabidopsis thaliana* plants. Being a *Brassicaceae* similar effects are expected to be achieved for closely related *Brassica* crops by treatment with *Bacillus*.

The aims of this project were to;

1. Develop *Arabidopsis thaliana* as a host plant for studies of beneficial plant-*Bacillus* interactions
2. Study the potential of *Bacillus* to promote growth of the host plant.
3. Study the potential of *Bacillus* to antagonize some common *Brassica* phytopathogens.
4. Identify some of the factors operating during plant-*Bacillus* interaction and pathogen-*Bacillus* interaction.



### 3 Materials and Methods

#### Bacterial and fungal strains and growth conditions

The *Bacillus amyloliquefaciens* subsp. *plantarum* strains UCMB5033, UCMB5036, UCMB5113 and FZB42 were maintained on LB agar (LBA) plates. The fungal strains were grown on potato dextrose agar PDA plates at 22 °C, 16/8 light and dark photoperiod (*Botrytis cinerea*, *Alternaria brassicae*, *Alternaria brassicicola*) or in darkness (*Verticillium longisporum* and *Sclerotinia sclerotiorum*).

#### Plant growth

*Arabidopsis thaliana* Col-0 and signaling mutants *coil-16*, *myb72*, *myc2*, *jar1*, *npr1* and *sid2* seeds were sterilized in 70% ethanol for 1 min, 10% bleach and rinsed three times with water. Seeds were germinated on MS agar plates and/or on soil in a growth chamber at 22 °C with 16/8 h photoperiod.

#### Isolation of antibiotic compounds

Antibiotic compounds were isolated from *Bacillus* UCMB5113 as described (Kim *et al.*, 2004). The HPLC eluted fractions were collected, pooled and lyophilized before being tested for antifungal activity. The chemical structures were determined by mass spectrometry.

#### Bacterial inoculation

The *Bacillus* strain UCMB5113 was inoculated 3 cm from the root tips and plates were kept vertically in a growth chamber for six days before recording the biomass (root and leaf). The level of the phytohormones IAA, CK, GA and brassinosteroids (BRs) were determined after bacterial treatment.

#### Bacterial volatiles

Effect of bacterial volatiles on plant growth

Seeds were germinated for four days on petri dishes containing 0.2x MS/0.6% Bacto agar and 1.5% of Sucrose. Later, the seedlings were moved to center partition plates containing 0.2x MS/0.8% Bacto agar/1.5% Sucrose. The plants were placed on one side and on the other side *Bacillus* strains were inoculated (bacterial strains were grown in TSB medium overnight) and sealed with tape. The plates were moved to a growth chamber at 22 °C with 16/8 photoperiod. Shoot fresh and dry weight was determined later. The volatile compounds produced by UCMB5113 on different media were identified by GC-MS analysis.

#### Effect of bacterial volatiles on phytopathogens

Fungal spores of *B. cinerea*, *A. brassicae*, *A. brassicicola*, *V. longisporum* and *S. sclerotiorum* were inoculated at the centre of the PDA plates. Fungal strains were then exposed to *B. amyloliquefaciens* UCMB5113 volatiles. Bacteria was placed in a small petri dish lid containing TSA and placed on the original PDA plate lid. The plate lid with bacteria down side and fungal strain up side, control plates were exposed to TSA without water and plates were sealed with parafilm. Plates were moved to a growth chamber at 22 °C with 16/8 photoperiod. The *V. longisporum* and *S. sclerotiorum* plates were incubated in dark. The inhibition of fungal growth was determined later.

## 4 Results and Discussion

### 4.1 *Bacillus* antibiotics (paper I)

*Bacillus* spp. are Gram-positive, spore forming and soil living bacteria. A number of *Bacillus* spp. have been found to suppress necrotizing pathogens/parasites or enhance plant growth (Ongena *et al.*, 2007; Ongena and Jacques, 2008; Kumar *et al.*, 2013; Elkahoui *et al.*, 2014; Rahman *et al.*, 2014; Farace *et al.*, 2014; Cawoy *et al.*, 2014). These *Bacillus* strains produce secondary metabolites, e.g. antibiotics and biosurfactants, that can restrict microbial growth serving in microbial antagonism to increase the competitiveness in complex substrates like soil. The majority of biosurfactants produced by *Bacillus* are LPs belonging to the surfactin, iturin and fengycin families that are genetically and biochemically well characterized and demonstrated to serve in plant colonization and direct antagonism to plant pathogens (Souto *et al.*, 2004; Ongena *et al.*, 2007; Ongena and Jacques, 2008; Rahman *et al.*, 2014; Farace *et al.*, 2014; Cawoy *et al.*, 2014).

#### 4.1.1 *Bacillus* effects on phytopathogens

In the present study, the isolated *Bacillus* UCMB5113 strain was tested towards several *Brassica* fungal phytopathogens (*B. cinerea*, *A. brassicae*, *A. brassicicola*, *V. longisporum* and *S. sclerotiorum*) on PDA plates. An antifungal activity was observed in the presence of *Bacillus* UCMB5113 bacteria or exudates but the effect was stronger with bacteria. This indicates that *Bacillus* UCMB5113 invest resources to constitutively produce antibiotic compounds that can diffuse into the surrounding environment to restrict other microorganisms (including phytopathogens) but that there also is an inducible effect on the production. However, the antagonistic effect against the fungal strains varied. Variation in antifungal activity has been reported also for other *Bacillus* strains (Nguyen and Kim, 2015; Rehman *et al.*, 2015; Kadaikunnan *et al.*, 2015). Further, a crude fraction isolated from *Bacillus* exudate enriched in

LPs showed high heat stability of antibiotic potency indicating high rhizosphere competence of UCMB5113. Subfractions of the LP were obtained by RP-HPLC and were examined for antifungal activity against phytopathogens on PDA plates. An antifungal activity was observed by the crude LP fraction and the RP-HPLC fractions four to ten showed varying antifungal activity against the different fungal strains. However, fraction 9 showed strong antifungal activity towards the tested pathogens.

#### 4.1.2 *Bacillus* effects on plants challenged with pathogens

In a pathogen bioassay where *Arabidopsis* leaves were treated with the *Bacillus* UCMB5113 crude exudate fraction disease protection was achieved. The protective effect may be due to both formation of a surface layer on the leaf and the content of antibiotics that prevents the attachment, multiplication and penetration of fungal spores, indicating a direct mechanism of protection.

Further, plant roots coated with crude LPs and grown on MS agar showed protection against *A. brassicicola* in *A. thaliana* wild type Col-0 and the mutant *sid2* but not in the mutants *coi1-16*, *jar1*, *myc2*, *myb72*, and *npr1*. The qPCR analysis showed significantly lower levels of *Alternaria* growth in wild type Col-0 treated with crude LP compounds compared to other treatments and mutants. Soil grown plants also showed restriction of fungal growth in Col-0 and *sid2* but not in the other mutants (*coi1-16*, *jar1*, *myc2*, *myb72* and *npr1*). The GUS reporter plants *VSP2:GUS* and *PDF1.2:GUS* proved the involvement of JA in the protection. These results suggest that *Bacillus* UCMB5113 can colonize the plants and stimulate systemic resistance in plants which is dependent on JA signaling pathways indicative of priming of ISR thus providing disease resistance through an indirect mechanism. It has been reported that *Pseudomonas* and some *Bacillus* strains stimulate ISR in plants independent of SA but dependent on JA and/or ET signalling (Verhagen *et al.*, 2010; Falardeau *et al.*, 2013; Rahman *et al.*, 2014; Farace *et al.*, 2014).

#### 4.1.3 Analysis of *Bacillus* lipopeptide structures

Mass spectrometry analysis of fraction 9 identified the main component as a novel linear form of fengycin LP. Previous studies have shown that *Bacillus* strains produce cyclic fengycins in nature (Liu *et al.*, 2014; Meena and Kanwar, 2015; Mora *et al.*, 2015) and not linear forms. To investigate the linear fengycin production by *Bacillus* UCMB5113, time course analysis was performed which showed that the production of the linear fengycins was constitutive and resulted in increased accumulation of compounds with time from day 1 to 6. This indicates that *Bacillus* UCMB5113 secretes linear fengycins but the biosynthetic route is not clear and post synthesis



modifications can be complex. LPs are formed by NRPSs organized as large multi-enzyme complexes (Strieker *et al.*, 2010) and it is difficult to predict the metabolism and post-synthesis modifications (Aleti *et al.*, 2015). For example changes in biotic or abiotic factors may change the final structure of LPs (Giessen and Marahiel, 2012).

Synthetic LP mimics of the fraction 9 linear fengycin compound with an acetyl (AcePEP) or a myristoyl (MyrPEP) group at the N-terminal was tested for antifungal activity against *A. brassicicola* and *V. longisporum* on PDA plates. Only the synthetic peptide with a fatty acid side chain, MyrPEP, showed antifungal activity. The qPCR analysis showed restriction of *A. brassicicola* growth in plants treated with MyrPEP.

The broad spectrum of LP produced by *Bacillus* UCMB5113 bacteria suggests efficient microbial antagonism with possibility to antagonise several microbes including phytopathogens through a direct mechanism which disturb surface properties of membranes. Further *Bacillus* UCMB5113 can also indirectly trigger ISR responses in the host plant against phytopathogens providing disease suppression or prevention. Based on these results we suggest that *B. amyloliquefaciens* UCMB5113 can be useful as a biocontrol agent against several *Brassica* phytopathogens. The broad spectrum of secondary metabolites with antibiosis effects, their high stability and direct and indirect protective effects support long term efficiency and improve rhizosphere competence.

## 4.2 Plant growth promotion by *Bacillus* (paper II)

The rhizosphere contains a huge and diversified microbial community, including PGPR. These PGPR enhance plant growth by direct or indirect mechanisms by production of phytohormones and other signals that change gene regulation and metabolism of the host plant that result in changed growth control manifested e.g. as a modified root system architecture.

### 4.2.1 *Arabidopsis* growth promotion by *Bacillus*

Plants inoculated with *Bacillus* UCMB5113 demonstrated increased biomass both of leaves and roots (increased branching, more root hairs) while the primary root was reduced compared to control plants. The increase of plant biomass was dose dependent. This indicates that *Bacillus* UCMB5113 produce metabolites which can alter the development of the plant root system, affecting meristematic activity differently among root tips, with decreased proliferation in the primary root and initiation of premature lateral root formation.

#### 4.2.2 Hormones and growth promotion by *Bacillus*

Previously, it has been reported that phytohormone producing bacteria can affect root system architecture by overproduction of lateral roots and root hair (Persello-Cartieaux *et al.*, 2003). Auxin is a phytohormone that plays an crucial role in promoting cell division (Enders and Strader, 2015; Ludwig-Müller, 2015) but in excess, inhibits cell elongation and increase the number of lateral roots and root hairs (Swarup *et al.*, 2007). Similar effects were reported in PGPR treated sugar beet seedlings, *Brassica juncea*, wheat and *Arabidopsis* (Loper and Schroth, 1986; Asghar *et al.*, 2002; Khalid *et al.*, 2004; Zamioudis *et al.*, 2013). The *DR5:GUS* reporter line of *Arabidopsis* treated with *Bacillus* UCMB5113 showed and enhanced auxin expression in the root cap, root meristem and procambium of roots. *Bacillus* UCMB5113 was also shown to synthesize auxins (as IAA) and the production was stimulated by presence of root exudates. Addition of *Bacillus* UCMB5113 or cell free exudate below root tips of vertically grown *Arabidopsis* plants resulted in growth arrest of the primary root tip or that the root tip avoided the bacterial samples growing in other directions. This indicates that *Bacillus* UCMB5113 secret IAA and/or other compounds with auxin activity that may interact with the plant hormone signaling and metabolism. It was shown that three PGPR *Pseudomonas* strains resulted in similar root system architecture of *Arabidopsis* as *Bacillus* UCMB5113 although one strain did not produce auxin indicating that PGPR production of auxins is not a prerequisite for the root effects observed (Zamioudis *et al.*, 2013).

The effect of *Bacillus* UCMB5113 on an *Arr5:GUS* transgenic *Arabidopsis* marker line showed enhanced expression of CK in plant roots, indicating activation of CK metabolism that may be involved in growth modulation. Variation in response of levels of different forms of GA and BRs were observed in plants root and shoots treated with *Bacillus* compared to controls. Apparently *Bacillus* UCMB5113 modulate the levels of different GAs and BRs that may contribute to the observed growth modulation.

#### 4.2.3 Role of plant signaling for growth promotion by *Bacillus*

The plant roots coated with crude LPs showed growth promotion in *A. thaliana* wild type Col-0, *coi1-16*, *jar1* and *npr1* but not in *myb72* on MS agar. Similar growth promotion was observed in plants grown on soil. This suggests that the corresponding genes are needed for growth promotion. However, variation of flowering among the treatments in Col-0 and mutants was observed. A significant increase of siliques, seed size and seed weight was observed in plants after repeated LPs treatment compared to water, methanol or LPs treatments. Previously it has been demonstrated that SA deficiency

stimulated leaf biomass and seed production (Abreu and Munné-Bosch, 2009). Crude LPs did not arrest primary root growth of *A. thaliana* plants compared to plants treated with bacterial exudates and synthetic IAA. This suggests that IAA like compounds were removed or lost its activity in crude LPs while processing.

Thus, plant-PGPB interaction seems to be distinguished by modulated hormone levels in diverse plants that result in stimulated growth. This study helps to understand some factors involved in the mode of action as a basis for further mechanistic studies.

### 4.3 *Bacillus* volatiles and their effects (paper III)

Plants emit photosynthetically fixed carbon in the form of VOCs from leaves, flowers, and fruits into the air, as well as from roots into the soil. These VOCs play key roles in attracting pollinators, support seed dispersion, and provide protection against pathogens and insects above and belowground (Peñuelas and Llusia, 2004; Raguso, 2008). On the other hand the rhizosphere microorganisms emit a blend of VOCs that may have crucial roles for interactions with plants, beneficial microbes, deleterious microbes, insects and nematodes (Wenke *et al.*, 2010; Hare, 2011).

#### 4.3.1 Effect of growth media on *Bacillus* VOCs and plant growth

In this study, the effect on *A. thaliana* growth was monitored by exposing plants to *Bacillus* strains on different media (TSA, LBA, M9A or MSA) in partition plates. A negative effect on plants was observed on TSA and LBA plates. Similar negative effects, no effect or positive effect was seen on plants roots when exposed to *Bacillus* UCMB5113 on different media. The growth varied among the *Bacillus* strains in different broth. The TSA and LBA media are rich in organic material and the bacteria grow faster and produced various metabolic VOCs that may be more susceptible for younger than older plants (Baillly and Weisskopf, 2012). Leaves of *A. thaliana* Col-0 plants were bigger after exposure to volatiles from *Bacillus* strains compared to control plants on MSA. A significant increase of fresh and dry weight of plants on MSA was observed and for dry weight on M9A. The growth promotion efficacy varied among the bacterial strains. It has been demonstrated that VOCs interact with plant cells and can change hormone levels, increase cell division and nutrient absorption (Zhang *et al.*, 2007; Xie *et al.*, 2009). The *Bacillus* strains showed differences in colony size and structure on different media. FZB42 grow rapidly and reached stationary phase earlier compared to other strains. Bacterial genes are involved in different metabolic processes for their growth and depending on media produce primary and secondary metabolites that can

vary in structure and effect. Bacterial operons related to metabolism express differently dependent upon the media used for their carbon source in a strain specific manner (Kierul *et al.*, 2015). The genome organization also show small but distinct differences among the *Bacillus* strains (Niazi *et al.*, manuscript).

Further UCMB5113 grown on MSA resulted in plant senescence at increasing doses but when grown on MSA plus root exudates where it instead increased fresh and dry weight of the plants. This shows that under *in vivo* conditions certain soil bacteria use root exudates as carbon source and that in turn can provide a positive effect on plant growth.

#### 4.3.2 Effect of *Bacillus* VOCs on pathogen growth

The fungal growth of *B. cinerea*, *A. brassicae*, *A. brassicicola*, and *S. sclerotiorum* but not *V. longisporum* was reduced in plates which were exposed to *Bacillus* volatiles. This shows the *Bacillus* strains produce antifungal volatiles which serve as fungicides. Microbial antagonism due to volatiles have been demonstrated in other cases suggesting this to be a common tool in nature to support survival in soil (Chuankun *et al.*, 2004; Blom *et al.*, 2011; Fiers *et al.*, 2013; Garbeva *et al.*, 2014). In the presence of root exudates the UCMB5113 volatiles showed antagonistic effect against *A. brassicae* and *V. longisporum* and the fungi lost mycelium pigmentation that may affect virulence (Liu *et al.*, 2005). GC-MS analysis identified several *Bacillus* UCMB5113 volatile compounds on different media. These compounds have in other systems been demonstrated to either have negative effect, no effect or growth promoting effects on plants, and/or inhibit fungal growth (Ryu *et al.*, 2003, 2004; Xiao and Xu, 2007; Kai and Piechulla, 2009; Blom *et al.*, 2011; Fiers *et al.*, 2013; Garbeva *et al.*, 2014). These results show that *Bacillus* strains produce volatile compounds that can increase plant growth and inhibit fungal growth, useful in agronomical application to improve crop yield and as a biocontrol agent to control phytopathogens.

## 5 Conclusions

It has been proven and demonstrated that PGPB can be potential microorganisms for enhancing plant growth especially under stress conditions. In the present scenario, experiments were made to screen *Bacillus* UCMB5113 against *Brassica* phytopathogens to elucidate their biocontrol mechanisms, and also the ability to promote plant growth through direct and indirect mode of action was assessed. *Bacillus* UCMB5113 showed direct antagonism to phytopathogens by production of antibiotic compounds and triggered ISR upon fungal inoculation and this resistance involved JA signalling steps. UCMB5113 increased biomass of *A. thaliana* Col-0 by at least by direct mechanisms. Finally it was observed that *Bacillus* volatiles resulted in growth promotion and inhibition of fungal growth under *in vitro* conditions illustrating the complex chemical interactions occurring during multi-organism interactions. Thus, the present study has shown that UCMB5113 is an efficient biocontrol agent in controlling *Brassica* phytopathogens in *Arabidopsis* through ISR activity and also promoting plant growth of high interest for implementation in agriculture.



## 6 Future perspectives

Plants are essential resources for human beings and other living organisms. Environment harmful chemical pesticides are used to control stress factors and improve crop production. Environmentally friendly strategies such as organic cultivation are necessary for crop production in the future. Methodologies for crop protection in organic productions are scarce throughout the world. Biocontrol is a tool with a potentially broad range of stress control and potential to improve crop production without the negative environmental impact associated with chemical pesticides. The main goals of this study were to characterize some of the effects of *Bacillus* interaction on plants and elucidate some of the mechanisms operating during *B. amyloliquefaciens* priming of plant defense against different stressors. Studies till date in this project have led to novel findings in the sustainable production area with emphasis of *Bacillus* promoting plant growth and antagonising pathogens.

Future research has to be focused on rhizosphere biology to create reliable unique settings to develop molecular and biotechnological approaches to increase the knowledge of the crucial molecules operating during plant-microbe interaction resulting in a beneficial interaction. Another challenging topic is to understand the microbial signals that elicit pathogen resistance in plants through ISR (or possibly other alternative pathways?). Techniques to exploit transcriptomics, proteomics and metabolics of plant-microbe interactions *in situ* in soil would be highly rewarding. In general a better picture of rhizosphere biology and biodiversity in relation to use of PGPR and BCA at scale is needed. The application of multi strain bacterial inoculation (“cocktails”) could be an effective approach to reduce harmful impact of stress on plant growth but prerequisites for effective combinations need to be established. The research so far carried out with bacterial volatile compounds (known and unknown compounds) could address the mode of action of different compounds and which combinations that are most effective. Volatile

compounds can be employed in agriculture as antibiotics/inducers against pathogens as illustrated by SOS signaling between plants and also the ability of certain plants to attract natural enemies. These studies provide a basis for further studies of mechanisms operating in beneficial plant-microbe interactions and also to develop potential methodologies to improve production of oilseed rape crops and other high value crops by more sustainable tools based on rhizosphere organisms as ecosystem services.



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