Studies of Plant Interactions with Other Organisms to Understand *Bacillus* Mediated Stress Management

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Cover: Cotton leaf worm (*Spodoptera littoralis*) larva feeding on oilseed rape (*Brassica napus*) leaf
(photo: Anna Johansson)

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Abstract

The naturally evolved plant defense system is not always effective, due to adaptations among the attackers. Crop protective chemicals have many negative effects on the environment. Ecosystem services, like beneficial microorganisms, are of great interest for plant stress management in sustainable crop production. In this study, the rhizobacterium *Bacillus amyloliquefaciens*, known to protect oilseed rape (*Brassica napus*) to fungal diseases, was investigated. The aims were to test whether stains of *Bacillus* could protect oilseed rape against insect herbivores, and to find out more about factors involved in plant defense and *Bacillus* mediated stress protection.

Depending on the mode of treatment *B. amyloliquefaciens* were able to protect oilseed rape plants against feeding by the generalist herbivore *Spodoptera littoralis*. Analysis of transcripts and hormones implied involvement of JA signaling in *Bacillus* interaction with oilseed rape, and metabolomic fingerprinting indicated special responses to the *Bacillus* treatment. Real-time PCR (qPCR) assays were developed for the closely related *B. amyloliquefaciens* strains UCMB5033, UCMB5036 and UCMB5113. Using this, we revealed that mainly roots are colonized, and we saw a genotype dependence of colonization and growth promotion efficiency on two oilseed rape cultivars. A test with feeding by one generalist and one specialist herbivore on gene silenced (virus-induced) and mutant *Arabidopsis thaliana* plants, revealed a complex role of the *MD2-related lipid recognition* (*ML3*) gene in defense signaling, affecting both jasmonic acid (JA) and salicylic acid (SA) associated responses. The plant protective ability of *Bacillus* was investigated using *A. thaliana* Col-0 and An-1, and differential disease suppression was found against a broad spectrum of pathogens but no mediated protection against a specialist herbivore. Analyses of defense genes, hormone levels and mutants indicated that UCMB5113 was capable of activating both SA and JA defense, dependent on NPR1. Overall, the studies carried out revealed some mechanism of *Bacillus* mediated priming of plant defense, involving resource allocation as part of a less costly defense strategy.

Keywords: Bacillus amyloliquefaciens, Oilseed rape, Arabidopsis, ISR

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Contents

List of Publications

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

I Ingela Fridborg, Anna Johansson, Johanna Lagensjö, Natthanon Leelarasamee, Kristýna Floková, Danuše Tarkowska, Johan Meijer, and Sarosh Bejai (2013). ML3: a novel regulator of herbivory-induced responses in *Arabidopsis thaliana*. *J. Exp. Bot.* 64(4), 935-948.

II Anna H. Johansson, Sarosh Bejai, Kristýna Floková, Danuše Tarkowská and Johan Meijer. Use of biotic and chemical agents for priming of oilseed rape (*Brassica napus*) defence against generalist insect herbivores. (manuscript).

III Anna H. Johansson, Sarosh Bejai, Adnan Niazi, Shahid Manzoor, Erik Bongcam-Rudloff and Johan Meijer. Establishment of strain specific quantitative PCR assays for closely related *Bacillus amyloliquefaciens* biocontrol agents and studies of plant colonisation. (manuscript).

IV Jesper Danielsson, Anna H. Johansson, Sarosh Bejai and Johan Meijer. *Bacillus* mediated disease suppression in *Arabidopsis thalian*a. (manuscript).

V Natthanon Leelarasamee, Sarosh Bejai, Anna Johansson, Kunling Chen, Shashidar Asari, Jonas Kjellin, Danuše Tarkowska and Johan Meijer. Multifaceted role of *Bacillus amyloliquefaciens* in priming of *Arabidopsis thaliana* defense responses against biotic stress (manuscript)

The contribution of Anna Johansson to the papers included in this thesis was as follows:

I Made all insect experiments, some of the QPCR analysis, the statistical analysis and reviewed and commented on the manuscript together with the co-authors.

II Experimental design, performed most of the experimental work and data analysis, wrote the manuscript together with the co-authors.

III Experimental design, most of the experimental work and data analysis, wrote the manuscript together with the co-authors.

IV Planning and execution of the colonization and growth promotion tests, data analysis, wrote the manuscript together with the co-authors.

V Made some of the experiments and all statistical analysis, reviewed and commented on the manuscript together with the co-authors.

Abbreviations

2,4-DAPG	2,4-diacetylphloroglucinol
ALMT1	*ALUMINUM-ACTIVATED MALATE TRANSPORTER1* (*Arabidopsis* gene)
An-1	Antwerpen (*Arabidopsis* ecotype)
BABA	Beta-aminobutyric acid
BABA-IR	Beta-aminobutyric acid-induced resistance
BTH	Benzothiadiazole
Can-0	Canary Islands (*Arabidopsis* ecotype)
Col-0	Columbia (*Arabidopsis* ecotype)
COR	Coronatine
CT	Threshold cycle
DAMP	Damage-associated molecular pattern
ET	Ethylene
ETI	Effector-triggered immunity
GUS	Beta-glucoronidase
HAMP	Herbivore-associated molecular pattern
HTI	Herbivore-triggered immunity
ISR	Induced systemic resistance
IST	Induce systemic tolerance
JA	Jasmonic acid
JA-Ile	Jasmonic acid conjugate with isoleucine
L*er*-0	Landsberg *erecta* (*Arabidopsis* mutant from Landsberg ecotype)
LPS	Lipopolysaccharide
MA	Malic acid
MAMP	Microbe-associated molecular pattern
NPR1	NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN1 (*Arabidopsis* protein)
PAMP	Pathogen-associated molecular pattern

PCA	Principal component analysis
PGPB	Plant-growth promoting bacteria
PGPR	Plant-growth promoting rhizobacteria
PDF1.2	PLANT DEFENSIN 1.2 (*Arabidopsis* protein)
PR	Pathogenesis-related
PRR	Pattern-recognition receptor
*Pst*DC3000	*Pseudomonas syringae* pv. *tomato* DC3000 (bacterial strain)
PTI	PAMP-triggered immunity
qPCR	Quantitative polymerase chain-reaction
ROS	Reactive oxygen species
SA	Salicylic acid
SIS	Systemic induced susceptibility
TF	Transcription factor
TMV	Tobacco mosaic virus
VOC	Volatile organic compound
VSP2	VEGETATIVE STORAGE PROTEIN 2 (*Arabidopsis* protein)
WIR	Wound-induced resistance

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1 Introduction

Plants cannot flee from the negative challenges they are exposed to, but have to stay and take the fight. They are therefore in need of an effective way to cope with all kinds of unfavorable conditions in nature; environmental factors like large shifts in temperature, deficiency or plenitude of water (Baker and Rosenqvist, 2004; Farwell et al., 2007), but also attacks from viruses (Balachandran et al., 1997; Guiterréz et al., 2013) and other harmful organisms (Dangl and Jones, 2001; Agarwal et al., 2006).

Wild plants in nature have through evolution, in an arms race with all surrounding organisms, developed defense mechanisms against more or less recognized attackers (Mauricio and Rausher, 1997; Karban and Agrawal, 2002), like parasitic plants (Parker, 1991; Bouwmeester et al., 2003), large grazing animals (Bryant et al., 1991; Bagachi et al., 2006), insect pests, nematodes, and pathogenic fungi and bacteria (Dangl and Jones, 2001). Plants have also formed alliances with other species, in the form of carnivorous enemies of herbivore insects (van Loon et al., 2000; van Oosten et al., 2008), and soil microbes lacking plant pathogenic traits (Reva et al., 2004; Kloepper et al., 2004; Verma et al., 2007; Barea et al., 2002). The latter may have the ability to compete with, or kill, plant parasites and pathogens in the soil, promote plant growth, or even boost the plants' own defense to become more efficient and specific.

Plant beneficial bacteria in the soil are called plant growth promoting bacteria (PGPB) or more specifically for those living in the soil nearest the root; plant growth promoting rhizobacteria (PGPR) (Saharan and Nehra, 2011; Schwachtje et al., 2012). PGPB promote plant growth through one of many or a combination of mechanisms, either directly or indirectly. Several different strategies for promoting plants have been discovered, such as suppression of plant disease (bioprotection), improved nutrient availability (biofertilization), or production of phytohormones (biostimulation) (Saharan and Nehra, 2011).

Direct regulation of plant physiology can be achieved by bacteria mimicking synthesis of plant hormones or those that make minerals and nitrogen more available in the soil, like the leguminous symbionts *Rhizobium* (Hirsch and Kapulnik, 1998; Saharan and Nehra, 2011). Indirect boost of plant growth or fitness can be provided through production of siderophores, or volatiles like 2,3-butanediol and acetoin or different antibiotic compounds; or through induction of plant-mediated induced systemic resistance (ISR) (Saharan and Nehra, 2011).

The PGPB are mutual symbionts with the host plant utilizing the plant resources (Manjula and Podile, 2001; Chen et al., 2007). Plants can form many different types of relations to other organisms, especially microbes, and can in some cases even shift from one type into another depending on the environment (Hirsch, 2004). Commensalism, symbiosis with one winning organism can turn into mutualism, where both organisms profit on the interaction (Hirsch, 2004).

Another example of mutualistic symbiosis is formed with mycorrhiza fungi, where the fungus helps the plant to utilize phosphorus in exchange for carbon (Hirsch, 2004; Meyer et al., 2012; Sharma and Yadav, 2013). Some mycorrhiza-plant relationships also offer stress tolerance to the plant, for example in high salinity soil (Porcel et al., 2012), or defense against nematodes (Hajra et al., 2013; Vos et al., 2013) and pathogens (Ahmed et al., 2013; Mosquera-Espinosa et al., 2013; Maya and Matsubara, 2013). Also *Thrichoderma* fungal species, used as biocontrol, provide benefits on plant growth such as promoting plant growth, and increasing the nutrient uptake from the soil (Sharma et al., 2011).

1.1 Basic Plant Defense

A basic form of defense is constantly present in the plant. This includes many different ways to try to hinder wounds, diseases or lowering of fitness.

1.1.1 Direct Defense

The always present constitutive defense includes phenotypic features like spines and trichomes that can inhibit feeding (Fernandes, 1994), strategies of growth and life cycle to avoid impact of different stresses, chemicals like secondary metabolites that can act as toxins or make the tissues less digestible. One strategy to overcome wounds caused by generalist herbivores is to allocate resources towards tissues that are not under attack (Schwachtje et al., 2006; Orians and Thorn, 2011). For example feeding by nicotine specialist herbivore *Manduca sexta* larvae alters resource allocations in *Nicotiana attenuata* plants,

through the action of a plant kinase SnRK1, towards the roots so that the plants become more tolerant to the feeding of the leaf tissues (Schwachtje et al., 2006).

Some strategies of defense are induced upon a stress challenge and the corresponding signal is then directed towards the problem. Attack-associated mechanical and chemical signals that are recognized by the plant can initiate reactions, making the plant more tolerant or resistant to disease or consumption. The induced direct defense against insects involves production of toxins and feeding deterrents (Chen, 2008; van Oosten et al., 2008), while the defense against pathogens can involve killing of tissue, or production of reactive oxygen species (ROS), like superoxide or hydrogen peroxide to strengthen the cell walls and prevent spreading and also signaling (O'Brien, 2012.).

The plant can sense an insect attack in several different ways, and the physical contact is the initial cue. Insect herbivores also have plant recognized elicitors located in the saliva and in egg-fluids (Wu and Baldwin, 2010; Arimura et al., 2011; Erb et al., 2012), which in most cases trigger the plant defense against the present attacker. Herbivore-associated molecular patterns (HAMPs) and plant produced damage-associated molecular patterns (DAMPs) are recognized and detected by plant pattern recognition receptors (PRRs), and eventually this leads to herbivore-triggered immunity (HTI) or wound induced resistance (WIR) (Erb et al., 2012).

Like the insect associated elicitors, microbes have plant recognized surface patterns called microbe associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs) (Erb et al., 2012). Examples of these are bacterial lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane (Dangl and Jones, 2001.), and flagellin which is a globular protein that form the filament in bacterial flagella (Gómez-Gómez and Boller, 2000). These are, like in the case of insects, sensed by the plant through PRRs, leading to PAMP-triggered immunity (PTI) (Erb et al., 2012).

Many stress situations make plants release a variety of volatile organic compounds (VOCs) into the surrounding atmosphere (Holopainen and Gershenzon, 2010), and some of these can act directly against herbivores. VOCs can also facilitate recovery from abiotic stress (Holopainen and Gershenzon, 2010).

1.1.2 Indirect Defense

Grazing or egg-laying by insects can lead to the release of plant VOCs, as an indirect way for the plant to resist pests, as some of these compounds attract carnivores that are interested in feeding on the herbivores (Hilker et al., 2001;

van Oosten et al., 2008; Holopainen and Gershenzon, 2010; Pineda et al., 2010).

Some soil microbes recruited by the plant and living in the plant rhizosphere can secrete antibiotic compounds, which can be beneficial for the plant. This can indirectly provide a defense against soil-living attackers. An example of this is the *Bacillus amyloliquefaciens* strain FZB42, which has many genes proven to be involved in the synthesis of lipopeptides and polyketides with nematocidal, antifungal and antibacterial activity (Chen et al., 2009).

1.2 Stress and Disease Causal Agents

Most herbivores and pathogens are opposed through the naturally evolved plant defense, but this is not always effective. Some co-evolved organisms have overcome this defense, making the plant unable to stay healthy; some have even become specialized on certain plants and are dependent on these for nutrients and reproduction.

1.2.1 Insects

Some insects have evolved molecules that can suppress the HTI and WIR strategies in the plant.

Herbivores that are specialized on specific plants for food, like *Plutella xylostella* on glucosinolate containing plants, have evolved mechanisms to overcome this defense including detoxification of secondary metabolites (Wittstock et al., 2004) and manipulation of the host defense (Erb et al., 2012). By releasing chemical signals that are associated with attackers of a different kind, some insects trick the plant into using a defense strategy that is ineffective and which can even lead to a down regulation of the mechanisms that would have a relevant effect (Bruessow et al., 2010).

Different insects have different feeding strategies, which means they have to be fought in different ways, for example chewing larvae and phloem feeding aphids (Pineda et al., 2010)

1.2.2 Pathogens

The virulence of many pathogens is derived due to interference with host defense responses. Effector proteins from the pathogen can suppress the PTI and therewith facilitate the pathogenicity of microbes (Lakshmanan et al., 2012). A bacterial effector, coronatine (COR), is known to increase virulence of *Pseudomonas* by inducing a response of jasmonic acid (JA) which antagonizes activation of salicylic acid (SA) signaling (Tsai et al., 2011),

which would otherwise make the plant capable of reducing the infection. COR has a similar structure as the JA conjugate with amino acid isoleucine (JA-Ile), which is involved in the activation of JA responses (Katsir et al., 2008). This is a case of systemic induced susceptibility (SIS) (Cui et al., 2005).

PTI is a relatively weak immune response that occurs when the host plant does not recognize the pathogenic effectors which damage the plant or modulate its immune response. Some plants have evolved resistance (R) proteins that specifically recognize pathogen effectors, resulting in effector triggered immunity (ETI) (Erb et al., 2012). Also pathogens have different strategies of infecting their host plants; some are necrotrophs killing the tissue before consuming, others are biotrophs feeding on live tissues (Spoel et al., 2007; Spanu and Kämper, 2010; Laluk and Mengiste, 2010).

1.3 Crop Production

The history of edible plants and humans is a long story and our need for plants as food resource, both for ourselves and for livestock animals have made us seek ways to protect crop plants from yield-decreasing circumstances. One way has been to cross plant individuals having beneficial traits, like strength and stability, and decease resistance, in order for these qualities to be inherited and improved for each new generation (Tester and Langridge, 2010). Other ways to fight unwanted insects and disease causing microbes have been to use chemicals that are pesticidal or antimicrobial, and the use of pesticides has for decades been the norm in conventional crop production (Kemi, 2006).

1.3.1 Brassicaceae crops

The family Brassicaceae includes many important crops, like oilseed rape, mustard, cabbage, cauliflower, broccoli, and turnip. They all contain glucosinolates, which are secondary metabolites that are degraded by the enzyme myrosinase. Both the glucosinolates and their degradation products are important for various processes in the plant, such as defense, biofumigation, and plant development, and they are also important for the interactions with other organisms (Rask et al., 2000). These organisms, like insects, pathogens, mycorrhiza, and other soil microbes, can be either harmful or beneficial to the plant. Some of the degradation products from glucosinolates, like isothiocyanates, are toxic to many generalist herbivores. Several glucosinolates are known, but each plant species has a unique mix of a subset of these (Halkier and Gershenzon, 2006).

1.4 Priming

Some of the plant signals induced by an attacking organism can also be transported from the tissues under attack to non-attacked distal leaves, giving a systemic effect on defense. Priming of plant defense means that the plant is prepared for a more rapid and accurate response, when exposed to future stress challenges (Conrath, 2011). The benefit of priming is thought to be a lower energy cost, as compared to a constitutively fully expressed defense response or a defense completely induced at the moment of an attack (van Hulten et al., 2006). Priming should be preferable for agricultural crops, since plant protection can be achieved without loosing too much of the yield (Bhattacharyya and Jha, 2012), and it would also have less negative impact on the surrounding environment.

An example of a priming induced by plant–plant signaling mechanism has been observed in native tobacco (*N. attenuata*) as *M. sext*a caterpillars fed on plants previously exposed to clipped sagebrush (*Artemesia tridentata tridentata*), leading to an accelerated production of trypsin proteinase inhibitors causing digestion difficulties to herbivores (Kessler et al., 2006).

SAR is a system, mostly triggered by biotrophic pathogens, which leads to an elevated defense throughout the plant, and SAR-induced resistance is effective against a wide range of pathogens (Durrant and Dong, 2004). The mechanism is mediated through the plant hormone SA, which is involved in the formation of pathogenesis-related (PR) proteins (Durrant and Dong, 2004). In the model plant *Arabidopsis thaliana*, the initial recognition signal activates a molecular transduction pathway, including the accumulation of endogenous SA and expression of the *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1* (*NPR1*) gene, which downstream leads to the induction of the PR-expressing genes (Durrant and Dong, 2004). NPR1 is an SA receptor under redox control (Mou et al., 2003; Lindermayr et al., 2010; Wu et al., 2012). Many of the targets of NPR1 in *Arabidopsis* belong to a group of transcription factors (TFs) called WRKY-TFs that are in turn involved in feedback regulation of the synthesis of SA (Fu and Dong, 2013).

Also in *Arabidopsis*, a mobile metabolite, the nine-carbon dicarboxylic acid azelaic acid accumulates in the vascular sap as a result of bacterial infection (Jung et al., 2009). This is a part of the SA induced priming for defense against pathogens like *P. syringae* pv. *tomato* DC3000, especially important in the systemic spreading of the immunity as it induces a gene, AZELAIC ACID INDUCED 1 (*AZI1*), encoding a protein important for vascular spreading of disease resistance (Jung et al., 2009).

1.4.1 Abiotic Inducers of Plant Priming

Beta-aminobutyric acid (BABA) is a non-protein amino acid, which has been involved in many studies of plant protection against many different challenges (Pineda et al., 2010; Conrath et al., 2006). BABA root drench treatment has shown a positive effect on many Brassicaceae species in their defense, to both generalist and specialist insect herbivores (Hodge et al., 2006), and also to pathogens (Tsai et al., 2011).

BABA induced priming for defense works through a specific pathway, called BABA-induced resistance (BABA-IR). Some of the mechanisms of BABA-IR-related defense to abiotic stress, and to certain pathogens, have been elucidated and shown to be associated with SA signaling and PR proteins (Zimmerli et al., 2000, 2001; Si-Ammour et al., 2003; Ton et al., 2005).

Benzothiadiazole (BTH), an SA-analogue, is a so-called plant activator and protects plants from diseases by activating the SA signaling pathway. The BTH- and SA-inducible WRKY TF genes that are induced by BTH treatment have been identified, and one of them, *WRKY45*, in rice (*Oryza sativa*) could enhance resistance in rice to rice blast fungus (Shimono et al., 2007). In *A. thaliana* WRKY TFs act in the SA signaling pathway through NPR1 (Shimono et al., 2007).

1.4.2 Biotic Inducers of Plant Priming

Tobacco mosaic virus (TMV) has been shown to induce SAR in tobacco plants leading to a development of increased resistance to further infection in systemic tissues (Durrant and Dong, 2004).

There are several studies showing that *Pseudomonas* and *Bacillus* trigger plant defense against varying forms of stress. *P. syringae* pv. *tomato* DC3000-induced SAR priming of *A. thaliana*, reduced growth and development of the generalist herbivore *Spodoptera exigua*, but was less effective against the specialist herbivor *Pieris rapae* (van Oosten et al., 2008). *Pseudomonas*-induced SAR has also been shown to be effective against many pathogens (Katagiri et al., 2002).

1.5 ISR

As mentioned previously, plant defense can also be triggered by some of the beneficial microbes colonizing the roots, such as PGPB and mycorrhizal fungi. Beneficial rhizobacteria have evolved strategies to suppress the host defense response, in order to colonize the root and set up a host-mutualistic association (Lakshmanan et al., 2012). Microbial priming of plant defense to insects has

been shown mainly using non-pathogenic *Bacillus* and *Pseudomonas* bacteria (Pineda et al., 2010).

ISR is the term used for improved plant defense induced by non-pathogenic microbes (Pineda et al., 2010; Saharan and Nehra, 2011), or induced systemic tolerance (IST) working as protection to abiotic stress (Yang et al., 2009). ISR has shown to be effective for many plant species and against different forms of attack, like insects, nematodes, viruses, fungi, and pathogenic bacteria (Pieterse et al., 1996; Pineda et al., 2010). ISR is a form of priming of defense, where genes show a systemic effect of increased expression in attacked leaves, and the mechanisms for this are thought to involve JA and ethylene (ET) responses (Pieterse et al., 1996; Pineda et al., 2010).

Non-pathogenic *Pseudomonas fluorescens* WCS417r-induced ISR in *Arabidopsis* is effective against different types of pathogens and insect herbivores (Pozo et al., 2008). It has been shoen to be effective against generalist tissue chewing insect *S. exigua*, but it is not effective against the specialist herbivore *P. rapae* (van Oosten et al., 2008).

One fungal species used as biocontrol in plant cultivation is *Trichoderma* spp., which apart from direct antifungal abilities (mycoparasitism) also has the skill of inducing JA/ET-based ISR in plants (Samuels, 1996; Chet et al., 2006; Sharma et al., 2011),

1.5.1 Mechanisms of ISR

Many studies have been made on microbe generated ISR, mostly in *Arabidopsis*, in order to rule out the genes and molecules involved in the different pathways. The primed state of these mechanisms can be differently effective against different types of challenges.

Some members of the protein family TIFY in *Arabidopsis*, have been shown to be transcriptional repressors involved in the regulation of ISR; these proteins have a jasmonate ZIM-domain, thereof the name JAZ (Staswick, 2008). JAZ binds to and hinders the TF MYC2 from regulating the JA signaling of ISR. Wounds, or pathogenic attacks can lead to an elevated level of JA-Ile (Chung et al., 2009), which promotes the binding of a the a COI1-SCF-complex to JAZ and this in turn leads to the degradation of JAZ and eventually a functioning ISR expression (Staswick, 2008). It has also been suggested that MYC2 is involved in a negative feedback loop of JAZ (Chico et al., 2008).

In the case of *P. fluorescens* WCS417r induced priming in *Arabidopsis* to *P. syringae* pv. *tomato* DC3000, the majority of the primed genes were regulated by JA or ET signaling (Verhagen et al., 2004). Two of the involved genes were the JA-responsive gene *VSP2* and the JA- and ET-responsive gene

PDF1.2, but the expression of these genes were not increased after only treatment with *P. fluorescens* WCS417r, while this caused up-regulation of *MYB72* in the root (Verhagen et al., 2004). In this way, priming of pathogen-induced genes allows the plant to react more effectively to the invader encountered without constitutive expression of the defense. Rhizobacteria-mediated ISR caused by *P. fluorescens* WCS417r bacteria gave transcriptional responses of 97 *Arabidopsis* genes locally in the rots, but no systemic effect in the leaves, while *P. syringae* pv. *tomato* DC3000 inoculation of WCS417r-induced plants, showed an elevated expression pattern of 81 genes in ISR-expressing leaves (Verhagen et al., 2004). The *P. fluorescens* WCS417r-induced ISR in *Arabidopsis* against the generalist tissue chewing insect *S. exigua*, works through a potentiated expression of the defense-related genes *PDF1.2* and *HEL* (van Oosten et al., 2008). *P. fluorescens* WCS417r colonization of *A. thaliana* roots, trigger ISR through the activation of an R2R3-MYB-like TF gene, *MYB72*, which is essential but not sufficient by itself to establish broad range ISR, acting upstream of ET in the ISR pathway, both being required in the roots during early signaling steps of rhizobacteria-mediated ISR (van der Ent et al., 2008). Microarray analysis displayed a overrepresentation of MeJA responsive genes in *P. fluorescens* WCS417r-mediated ISR-expressing plants and MYC2 has been shown to play a central role in JA- and abscisic acid-regulated signaling and has been described as a potential regulator in priming for enhanced JA-responsive gene expression during rhizobacteria-mediated ISR (Pozo et al., 2008).

Some strains of *P. fluorescens* produce a polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), making them very effective as biocontrol agents against many pathogens on *A. thaliana* and various crops (Weller et al, 2011). Due to results from mutant studies of the genes *npr1-1*, *jar1*, and *etr1* in *A. thaliana*, 2,4-DAPG is believed to be a part of the ISR induced by the 2,4-DAPG-producing *P. fluorescens and that* these bacteria operate through the ET/JA-dependent signal transduction pathway (Weller et al, 2011).

The rhizobacterium *Pseudomonas chlororaphis* O6 has the ability to induce ISR in *Arabidopsis* against *Botrytis cinerea*, and a study O6 colonisation, especially in combination with pathogen infection, lead to increased expression of a galactinol synthase gene (*AtGolS1*) and that this was mediated through the JA-dependent pathway (Cho et al., 2010).

The non-pathogenic rhizobacterium, *Pseudomonas putida* LSW17S can prime *Arabidopsis* Col-0 plants for *NPR1*, ET, and JA dependent disease resistance, for more than ten days, against *P. syringae* pv. *tomato* DC3000, and the priming is combined with accumulation of hydrogen peroxide or callose (Ahn et al., 2007). LSW17S can also elicit systemic protection against

pathogens like *Fusarium oxysporum* f. sp. *Lycopersici* or *Pseudomonas corrugata* (wilt, pith necrosis) in *tomato* (*Lycopersicon esculentum* L.) (Ahn et al., 2007).

The rhizobacterium *Bacillus cereus* AR156 has been shown to induce ISR via NPR1 simultaneously activating the SA and JA/ET signaling pathways in *Arabidopsis* Col-0 (Niu et al., 2011). A similar role for NPR1 has been shown in *Paenibacillus alvei* K165 mediated ISR against *Verticillium dahliae* (Tjamos et al., 2005).

A root colonizing rhizobacterium, *Bacillus subtilis* FB17, was shown to enhance defense to stomata-mediated entry of pathogenic *P. syringae* pv. *tomato* DC3000, in *A. thaliana*, by influencing two signaling pathways controlled by ABA and SA respectively, and thereby cause stomata closure (Kumar et al., 2012). An analysis of FB17 priming of defense in *A. thaliana* indicated that the resulting resistance to *P. syringae* pv. *tomato* DC3000 occurs via NPR1 and requires SA and ET, but not JA (Rudrappa et al., 2010).

1.5.2 Cross-talk

Jasmonates (JAs), ET, and SA are all important plant hormones with regulatory roles in induced defense against harmful pathogens and insects. Their signaling pathways are interconnected, providing the plant with a great regulatory potential to tailor its defense response to the invader encountered (Leon-Reyes et al., 2009). Defense in the form of SAR, is usually regulated by SA, while ISR is associated with JA. A negative cross-talk effect of the SA regulated pathway on the JA regulated ISR pathway has been reported (Spoel et al., 2003; Kunkel and Brooks, 2002; Koornneef and Pieterse 2008). The regulatory plant protein NPR1 is a key component required for both SAR and ISR, but *Npr1* transcript levels are not elevated in plants simultaneously expressing both types of induced resistance, which means that the normal level of NPR1 is probably sufficient to facilitate simultaneous expression of SAR and ISR (van Wees et al., 2000). It was also seen that a simultaneous activation of SAR and ISR could result in an additive effect on the level of induced protection against *P. syringae* pv. *tomato* DC3000 (van Wees et al., 2000), but at the same time NPR1 in *Arabidopsis* was demonstrated to be required for the SA-mediated suppression of JA-dependent defenses (Leon-Reyes et al., 2009).

The antagonism between SA and JA signaling is thought to function as a mechanism to fine-tune defenses that are activated in response to different or simultaneous attackers (Leon-Reyes et al., 2009). This negative regulation of JA signaling in *A. thaliana* is shown to be connected to the transcription of JA-responsive marker genes, *PDF1.2* and *VSP2*, being very sensitive to

suppression by SA, this due to a transient increase in glutathione levels (Koornneef et al., 2008).

ET has been suggested to be responsible for the NPR1 involvement of the SA-JA antagonism, possibly through enhanced allocation of NPR1 to function in SA-dependent activation of *PR* genes (Leon-Reyes et al., 2009).

The COP9 signalosome (CSN) is a protein complex (in *A. thaliana* and *tomato*) known to be involved in plant development. It regulates the activities of cullin-RING E3 ubiquitin ligases (CRLs), which in turn ubiquitinate proteins to target them for proteasomal degradation, and CSN has also been found to have an effect on plant defense responses to challenges like; mechanical wounding, attack by *M. sexta* larvae, and necrotrophic fungal pathogen *B. cinerea*, the effect seemingly linked to JA-related pathways, since silencing of CSN led to an increased expression of PR genes and a reduced synthesis of JA (Hind et al., 2011).

The previously described *P. syringae*-elicited SIS caused by the production of COR, may be a consequence of the mutually antagonistic interaction between the salicylic acid and JA signaling pathways (Cui et al., 2005).

1.5.3 Factors Influencing the Bacterial Colonization

Plants probably first recognize the beneficial microbes as invaders, but eventually tolerate them and allow them to colonize their root system (Zamioudis and Pieterse 2012) or even let them become endophytic (Compant et al., 2010). The plant thus has to, besides distinguish between different pathogens, also be able to recognize and react differently to beneficial microbes than to pathogenic microbes. It has been shown that *A. thaliana* plants can stimulate ISR evoking bacteria when being infected by *P. syringae* pv. *tomato* DC3000 and the ISR mechanism itself also seems to be involved in development of a beneficial microflora in the rhizosphere (Doornbos et al., 2012).

Also the plant genotype can influence how the interaction between plant and soil microbe takes place and how well the priming effect is established. A selection of *Arabidopsis* accessions were used to study natural variations in defense strategies in order to identify genetic loci that are involved in priming. The survey showed that plants having an enhanced basal resistance against a necrotrophic fungus (*Plectosphaerella cucumerina*) and an herbivore (*S. littoralis*) also had responsiveness in gene expression of JA-induced *PDF1.2*, while plants being more resistant to a hemi-biotrophic pathogen (*P. syringae* pv. *tomato* DC3000) showed responsiveness in *PR-1* induction after SA treatment, and also had constitutively expression of defense-related TFs (Ahmad et al., 2011).

Many parameters affecting priming and being affected by priming are not well understood and there seem to be many factors to consider. Plant growth promoting *P. fluorescens* has been shown to have a positive effect on the phloem-feeding generalist aphid *Myzus persicae* feeding on primed *Arabidopsis* plants while the crucifer specialist aphid *Brevicoryne brassicae* was instead unaffected (Pineda et al., 2012). The feeding insects can even affect the plant to either express a part of the defense that does not affect them negatively or make the plant not react at all defensive (Pineda et al., 2012).

Plant root secretions influence the rhizosphere-soil and the organisms living there, (Walker et al., 2003; Bais et al., 2006). For example, the defense response in *A. thaliana* caused by the bacterial pathogen *P. syringae* pv. *tomato* DC3000, occurs via malic acid (MA) transporters and expression of the gene *ALMT1*, which leads to an increased level of MA titers in the rhizosphere (Lakshmanan et al., 2012). MA can help the plant to recruit beneficial microorganisms, as in the example of *A. thaliana* recruiting *B. subtilis* FB17 (Rudrappa et al., 2008). The plant growth stimulating efficiency of PGPR can be affected by soil nutritional conditions (Saharan and Nehra, 2011).

1.6 Future Perspectives

More environmentally friendly crop production is a necessity for the future. Organic production of Brassica crops is very low in Sweden, mostly due to limited and poor methods to organically control pathogens and pests. In the absence of resistant varieties, extensive use of pesticides is often necessary and this cannot be used for organically grown plants. Novel solutions for pest management are therefore needed to facilitate organic cultivation of crops like Brassicas. Biocontrol is a promising tool for controlling pests and pathogens in agriculture, which does not have the extent of negative environmental impact as many of the chemical control agents used. The results of my project have given more knowledge about *B. amyloliquefaciens* and its potential application as biocontrol agent especially in oilseed rape production.

2 Methods

2.1 Plants

For tests of *B. amyloliqefaciens* mediated priming, different cultivars of oilseed rape (*B. napus*) have been used. Mostly the spring cultivar Westar was used, being sensitive to infections and responsive to *Bacillus* (Danielsson et al., 2007). The commercial cultivars Oase (winter) and Ritz (spring) were used because of the previously observed effect of their root exudates on the growth of the *B. amyloliquefaciens* strain UCMB-5113 (unpublished data).

For *A. thaliana*, the ecotypes Columbia (Col-0) and Antwerpen (An-1), were used in combination with the pathogen *P. syringae* pv. *tomato* DC3000. The plants were grown in sterile soil in growth chambers with controlled environment (16/8 h L/D, 22/18°C, 200 µmol m^{-2} s^{-1} light) or in sterile conditions, *Arabidopsis* on MS medium and oilseed rape in paper bags (16/8 h L/D, 22/20°C, 110 µmol m^{-2} s^{-1} light).

2.2 *Bacillus* Cultures

The *B. amyloliquefaciens* strains FZB42, UCMB-5033, UCMB-5036, and UCMB-5113 and the *B. endophyticus* strain UCMB-5715^T were grown in LB medium at 28°C with agitation up to fourteen days. Spores were selected by heat treatment (70°C for eight minutes) and the spore concentration determined by viable count analysis. The stock solution was kept refrigerated until use.

For plant treatment, 10^7 spores ml^{-1} in water was used, giving disease protection (Danielsson et al., 2007, Bejai et al., 2009), while water treatment was used as control (mock).

2.3 Treatment for Priming of Plants

The plants were treated with *Bacillus* in one of three different ways. Seed-dip, where the seeds were immerged in bacterial solution for two hours in room temperature, spray treatment of plants, or soil drench, where the *Bacillus* solution was added into the soil near the plant root. For BABA treatment, spraying (approximately 1 ml/plant of 30 µM BABA) was used.

2.4 Insect Pests

Cotton leaf worm (*S. littoralis* Boisd., Noctuidae, Lepidoptera) egg sheets were kept in controlled environment and provided synchronized hatching, with larvae that were reared on artificial diet. First instar larvae were used in the feeding experiments, and second instar larvae for metabolomic and transcript analysis to create more leaf damage in a short time frame. The first instar seems most discriminatory what concerns food source quality.

Non-choice experiments with *Spodoptera* investigated larval fitness after feeding on whole plants or detached leaves. For challenge of intact plants one plant was put in a net cage together with one larva carefully applied to one leaf in controlled environment as described above. In leaf assays one carefully detached leaf was put alone in a small Petri dish together with one larva. The larva was weighed at different time points or after a long time feeding.

Choice experiments with *Spodoptera* were made to study insect preference. One carefully detached leaf from each treatment was put in the periphery of a larger Petri dish together with the same number of larvae as of leaves, placed in the middle. The number of larvae sitting on each leaf was recorded at different time points.

2.5 Pathogens and Inoculations

P. syringae pv. *tomato* DC3000 was grown in LB medium or Kings' B medium at 37°C or 28°C, centrifuged and the pellet resuspended to OD_{600} 0.02 (10^7 cfu ml-1). The fungal strains were grown on potato dextrose agar (PDA) at 22°C (*Alternaria brassicicola*) or 16/8 hour photoperiod at 21/16°C (*A. brassicae* 980:3 and *L. maculans* 1245). Spores were harvested and counted using a Bürkner chamber and adjusted to 10^7 spores ml^{-1}. The pathogens were applied to the plants to match their infection strategy.

2.6 Scoring of Pathogen Inoculated Plants

L. maculans and *A. brassicae* infection was scored after one week. Presence of necrotic lesions at the punctures was used to score plants as either infected or uninfected. Infection with *P. syringae* pv. *tomato* DC3000 was scored four days post infection and lesion size was measured on a four degree scale, 1 (<25%), 2 (>25%), 3 (>50%) and the last step 4 (>75% of the leaves infected).

2.7 Gene Expression Analysis

Samples frozen in liquid nitrogen were pulverized in 2 ml tubes containing steel beads using a tissue lyser (Retsch mill®). For leaf/root samples approximately 100 mg was used for RNA extraction and RNA quantified by fluorometric analysis using Qubit (Invitrogen). cDNA was synthesized using a qScript™ cDNA Synthesis kit (Quanta). Q-PCR was performed based on the SYBR GREEN based assay with a ROX qPCR Master Mix 2x (Maxima®). Threshold cycle (C_T) values from the reference gene Ubiquitin5 and APT1 for Arabidopsis and Actin for *B.napus* were used to normalize data. Normalized transcript levels of each gene were calculated and the relative levels of transcription were calculated using the 2 CT method (Livak and Schmittgen, 2001). Real-time PCR, qPCR, was run in BioRad MyIQ with the software BioRad iQ5. Standard curves were set up for all the *Bacillus* strains involved. Strain specific primers were used for different genes.

2.8 Histochemical β-glucoronidase (GUS) Reporter Gene Expression

GUS transgenic lines (*VSP2:GUS, PR1:GUS* and *PDF1.2:GUS*) were grown in soil for 3 weeks before being treated with *B. amyloliquefaciens* spore solution for 2 days and challenge with *Alternaria*. Samples were collected at 5 days post infection and stained for GUS at 37˚C for maximum 24 h and destained using 70% ethanol before microscopy.

2.9 Metabolite Fingerprinting Analysis

Leaves from four experimental groups were subjected to metabolomic analysis (Ward et al., 2003; 2010). The groups consisted of three-week old *B. napus* cv Westar; treated with *B. amyloliquefaciens* 5113 by soil drench and exposed to herbivory (6 hours), untreated and exposed to herbivory (6 hours), and 5113-treated and non-challenged. Control plants where untreated and non-challenged. Metabolite extraction was made from freeze-dried leaves and the

supernatant was used for ^{1}H NMR or HPLC-ESI-MS analysis. SIMCA-P 9.0 was used for principal component analysis (PCA).

2.10 Statistical Analysis

Bar graphs with standard deviation error bars and t-tests, were conducted in Micrsoft Excel software program. One-way ANOVAs with Fisher pairwise comparison were conducted, using Minitab 16 Statistical software. For the NMR and ESI-MS fingerprint data of expression patterns, PCA models were constructed.

3 Results

3.1 ML3: a novel regulator of defense responses in *Arabidopsis thaliana*

Screening of ML gene family upon herbivory by specialist *Plutella xylostella* and generalist *Spodoptera littoralis* identified ML3 to play a prominent role in herbivory induced responses. Herbivory bioassays showed that larvae of specialist herbivore gained more weight compared to the specialist. Virus induced gene silencing (VIGS) of ML3 expression in plants compromised in JA and SA signalling revealed a complex role of JA and SA dependent responses. Further testing of *ML3:GUS* lines upon herbivory showed a localized expression around the damaged aread (manuscript to be submitted). Expression of *ML3* on plants prior treated with *Bacillus* and exposed to *S.littoralis* showed an elevated expression compared to the water treated insect damaged plants.

3.2 *Bacillus* vs Generalist Herbivore *Spodoptera littoralis* Feeding

Several methods were used for *Bacillus* application onto the plants in the experiments regarding the generalist *S. littoralis* herbivore feeding on oilseed rape plants. For many of the methods, like spraying and seed dipping, the results were inconsistent and without significant effects in the expected direction, i.e. a lower larval weight after feeding on *Bacillus* treated plants. Only soil drench, where the *Bacillus* solution (strain UCMB-5113) was added into the soil, near the plant root, gave a significantly lower larval weight compared to the water treated control and the untreated control.

Plants sprayed with the chemical priming agent BABA did not result in any significantly differing weight gain of *Spodoptera* larva compared to control plants. Interestingly the combination of BABA spraying with *Bacillus* strain 5113 applied by soil drench gave intermediate effects of the isolated treatments, suggesting a negative interaction effect by BABA on the *Bacillus* stimulated protection to herbivory.

3.3 *Bacillus* Pre-treatment Effect on JA Responses upon Herbivory and Metabolite Fingerprinting

The *Bacillus* treated plants exposed to herbivory showed a four-fold higher *LOX2* expression compared to the control (untreated and unchallenged) plants. The plants not treated with *Bacillus* showed two-fold higher *LOX2* expression upon herbivory compared to the control plants (untreated and unchallenged). The *Bacillus* treated and non-challenged plants showed 30% lower transcript levels of *LOX2* indicating that bacterial treatment to the roots primes the leaves for higher expression of *LOX2,* suggesting improved capacity for JA biosynthesis, upon insect challenge.

MPK4 has been previously shown to regulate herbivore induced plant resistance. *Bacillus* treatment had no effect on the *MPK4* levels, *Spodoptera* feeding increased *MPK4* levels two-fold, while a combination of the two increased the levels four-fold indicating priming of improved capacity for defense.

Bacillus treatment without any exposure to herbivory, did not affect JA levels. Upon herbivory *Bacillus* pre-treated plants showed significantly higher JA levels compared to control. However, co-treatment of plants with *Bacillus* and BABA seems to attenuate JA levels compared to BABA alone. The levels of the biologically active conjugate JA-Ile were also significantly higher in the *Bacillus* treated plants upon herbivory compared to other pre-treatments. In contrast to JA, the co-treatment with *Bacillus* and BABA resulted in the same JA-Ile levels as BABA only upon herbivory.

PCA of NMR based metabolomics data showed a significant difference between insect exposed plants and the untreated control plants. Also the *Bacillus* treated plants, both with and without insect feeding, were significantly separated from both the untreated control plants and the non-primed insect-challenged plants. Major plant signals induced by *Bacillus* treatment and such plants fed upon by insects compared to the untreated challenged plants were glucobrassicin (indole-3-yl-methyl-glucosinolate), sucrose, choline, malate/citrate, glutamate, and alanine. Significantly lower levels of maltose and glucose, and some aromatics thought to be flavonoids were observed. Plant

signals induced by merely insect feeding were glucobrassicin, rhamnoside and possibly threonine. Decreased signals were the same as for *Bacillus* treated plants, without the maltose signal and instead including a decreased malate signal. In correlation to the NMR analysis, both negative and positive mode HPLC-ESI-MS metabolite analysis showed that both *Bacillus* treated and treated/herbivory challenged samples were separated from both non-treated herbivory challenged samples and the controls. The *Bacillus* treated and treated/challenged samples could not be separated. Sucrose and choline signals were found to be significantly decreased upon herbivory in the non-treated plants, whereas, the *Bacillus* treated plants upon herbivory showed an increase in choline level. Glucobrassicin and methoxyglucobrassicin signals were found to be elevated to a significant level upon herbivory in the non-treated plants compared to the *Bacillus* treated plants.

3.4 SS2 and RS2 Expression is Differentially Affected by Priming Agents and Herbivore Challenge

In order to further analyze resource allocation and metabolite diversion after use of priming agents and herbivory the expression of oilseed rape orthologs to *Arabidopsis SS2* and *RS2* in leaves were studied. *SS2* transcript levels decreased slightly in insect challenged wildtype leaves but no systemic effect was found. *Bacillus* treatment increased *SS2* levels with 30% while after herbivory levels decreased more than for the herbivore challenged controls. Transcript levels of *SS2* after BABA treatment and *Spodoptera* feeding were similar to the control. The combination of *Bacillus* and BABA gave similar effects as for the non-primed control plants.

RS2 expression in wildtype plants was up-regulated after *Spodoptera* challenge 8-fold and 4-fold in local and systemic leaves, respectively. *Bacillus* treatment caused a 11-fold increase in *RS2* levels and herbivory increased expression even more in local leaves (16-fold) while a strong down-regulation (5-fold) was observed in systemic leaves. BABA treatment had no significant effect on *RS2* levels, but followed by herbivory it gave increased *RS2* levels in local and systemic leaves, compared to control plants. The combination of *Bacillus* and BABA treatment increased *RS2* expression 3.5-fold and after herbivory a stronger up-regulation (22-fold) was noted than for any other treatment in the fed leaf while the systemic leaves showed 40% down-regulated expression.

3.5 Development of Specific qPCR Assays for *Bacillus amyloliquefaciens* Strains

Sequencing of the *Bacillus* genomes allowed us to choose unique gene sequences after gene annotation to design strain specific PCR reactions. For that purpose the *tetB* gene in *B. amyloliquefaciens* UCMB5113 was chosen together with the *trpE(G)* gene and the *yecA* gene for *B. amyloliquefaciens* strains UCMB5033 and UCMB5036, respectively. Oligonucleotide primers optimised for qPCR based on sybergreen detection were designed. Tests of gene product formation using PCR gave one singe band of the expected size upon electrophoretic analysis.

Standard curves for the different gene amplicons were developed and showed good linearity over a great dynamic range (50 fg - 50 ng total DNA). The slope of the standard curves (-3.425 to -3.557) showed good correlation coefficients (0.999 – 1.000) indicating accurate conditions for quantitation with efficiencies from 91-96%. Ct values around 30 cycles corresponded to 0.25-1 pg of DNA depending on the strain. The specificity of the amplification was verified by the post reaction melting curve analysis showing only one product with the expected melting temperature for all amplicons.

In order to validate the specificity of the amplicon formation, samples were spiked with purified bacterial total DNA from other strains. Even in the presence of a million fold excess of heterologous DNA the threshold Ct values remained the same providing an accurate estimation of DNA. Soil total DNA, representing a large variety of microorganisms, did not provide any amplification products further indicating the specificity of the amplification. Soil DNA had no inhibitory effect on the qPCR reaction.

3.6 *Bacillus* Disease Suppression in *Arabidopsis*

The *B. amyloliquefaciens* strains UCMB-5036 and UCMB-5113 provided significant protection of the *Arabidopsis* An-1 ecotype against both the hemibiotroph *L. maculans* and the necrotroph *A. brassicae*. This effect was a combination of a decrease in infection frequency and the degree of disease symptoms. The *Bacillus* strains UCMB-5715T and UCMB-5033 gave no protection with plants having yellow leaves and a senescence phenotype.

The *Bacillus* strain UCMB-5113 gave significant protection against the hemibiotrophic pathogen *P. syringae* DC3000 in both An-1 and Col-0 shown as a clear decrease of lesions and chlorosis. *Bacillus* UCMB-5036 also showed a tendency to reduce disease symptoms but this effect was not significant. An-1 was significantly more susceptible to *P. syringae* than Col-0.

3.7 Colonization of *Bacillus* on Oilseed Rape Plants and *Arabidopsis* An-1 and Col-0 Ecotypes

The *Bacillus* strains can colonize plant roots and protect the plant against abiotic and biotic stress. It is not known more exactly what parts of the plant the biocontrol strains colonize and to what extent.

To address this we used the specific qPCR assay to study the colonization of two oilseed rape cultivars by the UCMB5113 strain. Seeds were dipped in *Bacillus* solution and then allowed to germinate. The level of *Bacillus* (cfu count) initially decreased significantly on both oilseed rape cultivar seedlings. After two weeks cfu analysis indicated lower root colonization on both cultivars but higher on cv Oase than on cv Ritz, while very low levels were detected on the cotyledons. qPCR assay data were similar to the cfu counts when seeds and two day seedlings were analyzed. At 9 dai the qPCR indicated higher colonization of Ritz than the cfu count. At 14 dai the qPCR showed similar decreased levels of UCMB5113 in Oase and Ritz. *Bacillus* levels in cotyledons were very low based on qPCR analysis in accordance with cfu results.

To further test the qPCR assay we analyzed the levels of *B. amyloliquefaciens* 5113 in an experiment with priming of disease tolerance with *Arabidopsis* plants. Two *A. thaliana* ecotypes (L*er*-0 and Can-0) were soil drenched with *Bacillus* 5113 spores when they were three weeks old. Two days after *Bacillus* treatment, leaves were pressure infiltrated with the pathogenic bacterium *P. syringae* pv. *tomato* DC3000 and disease was apparent one week later. The analysis showed that the *Bacillus* amplified in the soil when the plants were developing and with somewhat higher levels for Can than L*er*. After bacterial challenge *Bacillus* levels were the same in the rhizosphere of Can plants while the level for L*er* had increased with almost 50%.

The *Bacillus* based qPCR, run with plant- and bacterial DNA extracted from *Bacillus* treated plants, showed a somewhat varied pattern of plant colonisation, both in terms of plant genotype and of *Bacillus* strain. All *Bacillus* strains showed, as expected, an increase over time from two weeks to three weeks after seed treatment, although not always of significance. Only the strain UCMB-5113 gave a significant difference in colonization between the two ecotypes where Col-0 had a higher number of bacterial DNA copies compared to An-1. This was observed for both time points.

3.8 Defense Related Gene Expression in UCMB-5113 Treated Leaves in *Arabidopsis* Wild-type (Col-0) upon Challenge with *Pseudomonas syringae* pv. *tomato* DC3000 or *Alternaria brassicicola*

To investigate the pattern of gene expression after treatment with UCMB5113 in *A. thaliana* challenged with *P. syringae* pv. *tomato* DC3000*,* qRT-PCR analysis was carried out for the SA inducible *PR1* and JA inducible *JAZ10* marker genes and *MYC2* transcription factor. A time course study revealed only at 24 h after challenge inoculation with *P. syringae* a 2.5 fold higher expression of *PR1* in the UCMB5113 treated plants compared to the water treated pathogen challenged plants. *JAZ10* transcripts were found to be up-regulated at an early time point of 3h post challenge with *P. syringae* compared to the UCMB5113 treated plants. At 24 h the *JAZ10* expression was found to be back to the basal level in the *P. syringae* challenged UCMB5113 treated plants. Even though *MYC2* was found to be induced at 3 hpi in the UCMB5113 treated plants it was not induced at any further time points. However the transcripts were observed to have an up-regulated trend at 6 h and 24 h in the water treated *P. syringae* challenged plants.

A time course qPCR analysis was carried out on the plants treated with UCMB5113 and challenged with *A. brassicicola. MYC2* was found to be 1.5 fold up-regulated upon challenge inoculation in the UCMB5113 treated plants. The JA marker genes *VSP2* and *PDF1.2* were induced at an early time point onwards upon pathogen inoculation in the UCMB5113 compared to the water treated controls. UCMB5113 treated plants upon challenge inoculation showed the highest induction at 6 h and a significant decrease at 24 h, whereas the *PDF1.2* expression was observed to be high even at 24 h.

3.9 UCMB-5113 Primes *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 and *Alternaria brassicicola* in a SA Independent and JA Dependent Manner

Previous studies (Danielsson et al., 2007; Bejai et al., 2009) showed that *B. amyloliquefaciens* mediates protection against a variety of fungal phytopathogens and seems to involve JA. To explore further the *Bacillus* induced resistance against the hemibiotrophic pathogen *P. syringae* and the necrotroph *A. brassicicola* bioassays were performed in the genetic background of JA, SA and ET deficient *Arabidopsis* plants (*etr-1, jar1-1, coi1-1,* NahG*, npr1-1*). Pretreatment with UCMB5113 to the roots of the plants before challenge inoculation led to a significant reduction in disease symptoms in all the signaling mutants expect *npr1-1.* At 5 days post inoculation

UCMB5113 pretreatment led to a significant reduction in pathogen density in the leaves of all the tested *Arabidopsis* signaling mutants except for *npr1-1* compared with the respective control. However higher pathogen density was observed in the *Bacillus* pre-treated NahG compared to the wild type Col-0. However, the ET signaling mutant *etr1-1* did not show any significance compared to the wild type. These results indicate that *NPR1* played prominent role in *Bacillus* mediated enhanced resistance to a bacterial and fungal pathogen in *A. thaliana* and not *ETR*.

UCMB5113 pretreatment before challenge inoculation with *A. brassicicola* decreased significantly the disease symptoms only in the NahG, *etr1-1* and the wild type Col-0. However UCMB5113 did not reduce disease symptoms in the *npr1-1, coi1-1* and *jar1-1* mutants.

3.10 JAZ1 is a Negative Regulator and MYC2 a Positive Regulator during UCMB5113 Mediated ISR

To examine further whether JAZ1 and MYC2 are involved in UCMB5113 mediated disease protection against *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000) and *A.brassicicola*, we analysed the effect in *jaz1-1* and *myc2-2* plants. UCMB5113 treatment reduced disease severity in Col-0 plants against *Pst*DC3000. However, the *jaz1-1* mutant showed a partial reduction in disease symptoms for *Pst*DC3000 in the UCMB5113 treated plants compared to the controls. The *myc2-2* mutants failed to develop protection against both the pathogens.

Bioassays were also carried out in the *pad3-1* mutant that is camalexin deficient and routinely used in ISR bioassays due to the susceptible nature to *A. brassicicola* (van der Ent al., 2008; Thomma et al., 1999; Ton et al., 2002). UCMB5113 treatment significantly reduced disease symptoms caused by *A.brassicicola* infection, whereas the *pad3-1*/*myc2-2* double mutants failed to mount protection against this pathogen. These above results indicate that both *JAZ1* and *MYC2* play important roles in ISR against pathogens of varied lifestyles.

Significantly enhanced expression of both *JAZ1* and *MYC2* was observed in the *Bacillus* treated plants. In the *npr1-1* mutants that have been previously shown to be unable to express an ISR response (Pieterse 1998), the expression of MYC2 was significantly reduced in the UCMB5113 treated plants. *JAZ1* was partially up-regulated upon UCMB5113 colonisation to the roots. These results provide evidence that UCMB5113 colonisation to the roots elevated expression of *JAZ1* and *MYC2*. Partial protection observed in the *jaz1-1*

mutants indicate that other JAZ family members might be playing a role in priming.

3.11 MYC2 Activation is not Compromised in *myb72* Mutants

Previously van der Ent et al., (2008) have shown the primary role of *MYB72* in activation of rhizobacteria mediated ISR in *Arabidopsis*. Our results also showed a significant up-regulation of *MYB72* transcripts in the roots of *Arabidopsis* Col-0 wild type treated with UCMB5113. To further understand if the MYB72 induced systemic resistance was through the activation of MYC2, we analyzed leaves of the *myb72-1* mutant after treatment with UCMB5113. Our results showed elevated levels of *MYC2* and *JAZ1* transcripts in the roots of *myb72-1* mutants treated with UCMB5113. This shows that the activation of MYC2 and JAZ1 in the systemic tissues after colonization by UCMB5113 is controlled by other unknown transcription factor(s) apart from MYB72.

3.12 Activation of SA and JA Signaling Components during *Bacillus* Colonization

We further investigated whether UCMB5113 triggers the basal level of JA and SA pathways during plant development. Two-week-old reporter plants for JA and SA signaling with GUS driven by promoters for VSP2, PDF1.2 or PR1 were root dip inoculated with UCMB5113 and stained for GUS expression. Two days after UCMB5113 treatment, a significant VSP2 expression was observed in the roots and leaves. Whereas PDF1.2 expression was restricted to the leaves, the expression was slightly down-regulated in the UCMB5113 treated plants. Interestingly a weak expression of PR1 was observed in the leaves compared to the water treated controls. One week after UCMB5113 treatment to the roots, VSP2 maintained a higher expression in both the roots and leaves compared to the control. No significant difference was observed in the PDF1.2 expression in both the controls and UCMB5113 treatments. Together these results indicate that UCMB5113 triggers defense responses in the plants.

4 Discussion

The main purpose of this doctoral project was to study the effectiveness and protective range for promising bacterial biocontrol candidates identified from earlier studies conducted with oilseed rape (Reva et al., 2004; Danielsson et al., 2007).

4.1 *Bacillus* Treatment against Insect Herbivory

The first part of the study was made to rule out whether *Bacillus* treatment of oilseed rape (*B. napus*) plants would result in an increased defense against insect feeding by the generalist herbivore *S. littoralis*. This was measured as effect on the larval fitness, i.e. the gaining of body mass after feeding.

B. amyloliquefaciens treatment of plants has previously been shown to mediate a JA-dependent effect of elevated defense mechanisms (Bejai et al., 2009), and this kind of defense has been shown to work against insect herbivore feeding (Wu and Baldwin 2010). The protective effect of *Bacillus* soil-drench against the invasive species *S. littoralis* observed here was thus not surprising. Earlier attempts in the project with *Bacillus* spray-application on the plants gave a less obvious effect, and one can suspect that efficient colonization of the plant roots is an important cue in the plant mediated effect, which has also been reported about PGPB biocontrol in agricultural production (Compant et al., 2010).

4.1.1 *Bacillus* Treatment versus a Plant Defense-inducing Chemical

A comparison of *Bacillus*-treatment and the defense-inducing chemical BABA was also made. BABA root treatment has earlier been shown to negatively affect larval weight of both generalist and specialist insect species (Hodge et al., 2006). In this study no such protective effect of BABA spray-treatment against *Spodoptera* larvae was seen. This might be due to the difference in

application technique, compared to the previously reported effect. An indication of a negative cross-talk between the SA-induced defense associated with BABA (BABA-IR) and the ET- and JA-induced defenses (ISR) connected to *Bacillus* priming was also seen here after combinatory treatment with the two. This result is in correlation with other observations (Bruessow et al., 2010; Pieterse et al., 2001.

4.1.2 Effect of *Bacillus* pre-Treatment and Herbivory on Plant JA-responses

The signal transduction pathways of plant defense are divided into different branches which are complexly connected and sometimes cross-talking. Simplified models have been made, in attempt to rule out what kind of stresses causes which branch to be activated. Herbivore-derived wounds have been put in a context of JA-mediated defense responses. Studies have indicated that some plant responses involve multiple hormonal pathways, to fine-tune the resulting action according to the present stressing factor. Plant defenses against insect herbivores and necrotrophic pathogens are mostly regulated by JA signaling, but through different branches of the pathway (Verhage et al., 2011).

In this study, JA response was measured in oilseed rape plants. This was done by studying the expression of a common JA response marker-gene *LOX2*. A two-fold higher expression was observed after *Spodoptera* feeding, which indicates an induced defense response. This correlates with previously reported *LOX2* expression in herbivore-exposed *A. thaliana* plants (Pozo et al., 2008; Chung et al., 2008; Bejai et al., 2012; Fridborg et al., 2013).

When we let *Spodoptera* feeding take place on plants that were pre-treated with *Bacillus*, an even higher *LOX2* expression was observed, which indicates a JA dependent priming effect. The effect we saw on *LOX2* seem to differ somewhat from that seen in studies of *Pseudomonas* mediated priming of *A. thaliana* (Pozo et al., 2008), since in our case *Bacillus*-treatment alone gave a slight decrease in *LOX2* expression pre-challenge. This decrease could be a result of plant response to the biocontrol bacteria, to facilitate root colonization. Similar tendencies have been observed in mycrorhizal colonisation on roots of *A. thaliana* (Stein et al., 2008).

MPK4 is involved in the transcript accumulation of JA responsive genes (Petersen et al., 2000; Brodersen et al., 2006), and acts as a positive regulator of JA and a negative regulator of SA in *Arabidopsis* (Petersen et al., 2000). Overexpression of *MPK4* has been shown to give *B. napus* plants enhanced JA-associated resistance to the necrotroph *Sclerotinia sclerotiorum* (Wang et al., 2009). In this study *Bacillus*-treatment followed by herbivory resulted in a higher transcription of *MPK4*, compared to the mock-treated plants.

4.1.3 Effect of *Bacillus* pre-Treatment and Herbivory on Plant Metabolism

We also examined the effect of *Bacillus* treatment and *Spodoptera* herbivory on oilseed rape metabolism. Previous studies of oilseed rape have shown that JA causes accumulation of indole glucosinolates (Bodnaryk 1994; Doughty et al., 1995). In this study *Spodoptera* herbivory induced indole glucosinolates as a direct defense response, but *Bacillus* treatment seemed to reduce this. This indicates a modified JA signaling. Earlier experiments with *Arabidopsis* lines that overexpress glucosinolate have showed differential feeding patterns for *S. littoralis* for different glucosinolates, but in that case indole glucosinolates were never tested (Bejai et al., 2012). Other studies of *Arabidopsis* lines that are deficient in indole glucosinolates though, have recorded an increased feeding (Schlaeppi et al., 2008). Our findings showing that *Bacillus* treatment apparently make plants avoid costly effects by reducing indole glucosinolates, in favor of other compounds, are supported by another recent report saying that PGPR can recruit JA responses and prevent indole glucosinolate accumulation (Zamioudis and Pieterse 2012).

The mechanisms involved in microbe mediated priming of plant protection are poorly understood and there is little information of the role of metabolites. The metabolite analysis conducted here did reveal some general patterns where the *Bacillus* treated plants (wounded or not) distinctly grouped together, apart from both control and insect damaged plants indicative of a priming mechanism and not classical (JA) induction of defense. Effects on both primary and secondary metabolism were found. The *Bacillus* treated plants had an increased content of sucrose, certain organic acids (malate and citrate), amino acids (glutamate and alanine) as well as choline. An increased content of sucrose is similar to previously reported plant survival strategies using changed carbon allocation (Schwachtje et al., 2006; Ibraheem et al., 2008). The idea is that, instead of a more direct defense in the form of elevated levels of glucosinolates, resource allocation towards the roots provides the plant with resources for growth once the insect has disappeared. Increased sucrose levels could also be linked to improved sensitivity to wounding and potentiated JA-related defense, since sucrose can serve as a self elicitor after wounding (Heil et al., 2012). Increased choline provides further resources for phospholipid biosynthesis or even osmolytes, but this seems less of a need after herbivory. The effects on acids may imply lower respiration and increased glycolysis and fermentation. Elevated levels of glutamate also provide the plant with many opportunities, due to the central role of glutamate and glutamine in plant metabolism. The decrease in maltose and glucose in primed plants after insect feeding suggests lower starch degradation and availability, and could be a strategy by the plant to make the leaf tissue less nutritious and appetizing for

the insect. Malate has been proven to be a factor in plants relation to beneficial microbes (Casati et al., 1999; Rudrappa et al., 2008). Citric acid has also been reported to be a key component in tomato root exudate, directing chemotaxis of beneficial *P. fluorescens* WCS365 (de Weert et al., 2002). Hence possibly, increased levels of malate and citrate may stimulate the *Bacillus* bacteria to become more active in supporting the plant. In a study of metabolic alterations in *Brassica rapa* leaves after herbivore attack, it was shown that the molecules most affected by the feeding for local and systemic leaves were alanine, threonine, glucose, sucrose, feruloyl malate, sinapoyl malate, and gluconapin (Widarto et al., 2006). It thus seems that lowering of sucrose and increasing glucosinolates may be a common strategy in *Brassicaceae* plant responses to herbivorous insects, while bacterial priming increase sucrose levels and attenuate glucosinolate induction as a result of *Spodoptera* feeding.

4.1.4 Effect of Priming and Herbivory on Plant Expression of SS2 and RS2

Analysis of *SS2* and *RS2* addressed effects on genes related to carbohydrate resources, transport and signalling as well as stress tolerance properties. Sucrose is the most important carbohydrate resulting from photosynthesis and a major transport carbohydrate in plants but can also stimulate anthocyanin production improving antioxidant defense. Changes in plant sugar levels as a result of exposure to pathogens or symbionts have effects on energy and carbon resource allocation but may also prime immune reactions (Moghaddam and van den Ende, 2012).

The stress induced changes in carbohydrate metabolism involve MAPKs, where we found *MPK4* to be upregulated more in *Bacillus* treated and challenged plants than for any other treatment. Raffinose has been implicated to be a major player for control of source-sink ratios of carbohydrates by phloem loading, vectorising the transport out from source tissues (Dinant and Lemoine, 2010). In addition, raffinose provides protection to abiotic stresses serving as osmoprotectant and scavenger of reactive oxygen species but has also been suggested to act as a signal to cells to support acclimation or cell death depending on the circumstances (Valluru and van den Ende 2011). The changes imposed by *Bacillus* pretreatment suggest that raffinose levels indeed are modulated by this priming agent as a mechanism to support the plant against any subsequent challenge. BABA treatment increased basal *RS2* expression somewhat but attenuated levels upon *Spodoptera* challenge compared to the non-treated plants. The increased transcript levels of RS2 observed in the BABA and *Bacillus* combination shows that *Bacillus* elicits the production of RS2 to attenuate the BABA induced SA responses. The mechanism of action of raffinose family oligosaccharides in plant defense is

still far from clear but an interesting observation is that the raffinose precursor sugar galactinol has been assigned a more direct role in priming. It has been suggested that galactinol functions as a signaling factor for *Pseudomonas chlororaphis* priming of ISR based on analysis of gene expression, mutant analysis and effects of galactinol addition to plants (Kim et al., 2008; Cho et al., 2010). Data from different sources thus support that raffinose family oligosaccharides have an important role in the improved plant defense observed after successful priming and this is probably the result of both direct and indirect effects on plant cells.

4.2 Strain Detection in Soil

More knowledge about the interactions between microbes and plants is necessary in order to optimize biocontrol use under natural conditions in agriculture, as the complex microcosm in soil will influence the conditions for the biocontrol agents (Ehrenfeld et al., 2005).

4.2.1 Specific Assays for Detection of *Bacillus amyloliquefaciens* Strains

Colony-forming-unit analysis on agar medium is a time consuming tool for determining bacterial presence. Also, problems with strain identification, overgrowth or competition, in complex microbiological backgrounds such as soil, make it sometimes less practical. Bacteria that have not formed spores might also be sensitive to storage, which could be necessary in large-scale experiments.

High levels of related, and potentially cross-reacting, DNA-sequences can be a problem. We wanted to develop a specific and sensitive assay for identification and quantification of our three *Bacillus* strains. A robust and reliable qPCR assay was needed for determination of their individual abundance on strain-level.

4.2.2 qPCR Assay

The qPCR assay tried here showed a million-fold dynamic range with detection of amounts down to 50 fg of total DNA giving 32 to 34 cycles, corresponding to 12 genome copies of bacteria based on genome size estimated to 3.9 MBp. This assay can be used with total DNA isolated from small samples, 0.25 gram soil or 100 mg plant samples, and the strain-detection tolerates million-fold excess of other DNA. Isolation and quantitation of DNA, and qPCR analysis can be run in the same day giving fast data generation.

4.2.3 qPCR Assays of *Bacillus* Colonization of Oilseed Rape Cultivars in Soil

The developed qPCR assay was in this study used to assess the colonization of oilseed rape after seed treatment. Both cfu and qPCR analysis of *Bacillus*-treated oilseed rape plants showed colonization of the roots on two different cultivars. A difference in the two detection methods was observed and this could be due to dead bacteria, not detectable on agar plates, or the bacteria around roots could be captured in extracellular structures, which could hinder colony formation (Hawes et al., 2012).

4.2.4 Conclusion of the Colonization study

Colonization was higher on young roots, maybe due to root exudation properties (Rudrappa et al., 2008) or unfavourable development under the axenic conditions. Also some genotype dependent differences in the bacterial colonization were detected. Mixed *Bacillus* strains could co-exist on the roots and the total number of bacteria encountered on roots was higher with the mixture, which means there is a possibility to use different kinds of biocontrol and growth promoting strains to give added values to the plant.

Colonization tests were also performed with *Arabidopsis* in sterile environment and a time dependent increase of *Bacillus* DNA on the plants was seen, which differ from the soil experiment with oilseed rape. Also, a significant difference was seen between two tested natural ecotypes. This kind of genetically dependent differences in biocontrol efficiency can probably also differ in artificially bred cultivars of crop plants. Also growth promotion provided by at least three of the *Bacillus* strains seems to be dependent on plant genotype.

4.3 Strain Dependence of *Bacillus* Priming

4.3.1 Effects of *Bacillus* Strains on Plant Protection against Pathogens

Bacillus UCMB-5036 and UCMB-5113 treatment of *Arabidopsis* were both effective *in vivo* against both *Alternaria brassicae* and *Leptosphaeria maculans*. The same result was seen *in vitro* against *L. maculans*. *Bacillus* UCMB-5113 gave an effect against *P. syringae*, but *Bacillus* UCMB-5715^{T} gave no protection against any of the tested pathogen. *Bacillus* UCMB-5033 was effective against *L. maculans* in our *in vitro* screen but showed no effect in soil.

These results are similar to those given in an earlier study with *B. napus* and the same *Bacillus* strains (Danielsson et al., 2007), where UCMB-5036 and UCMB-5113 also gave protection against the same pathogens. In that same study UCMB-5715^{T} gave no protection at all, while UCMB-5033 gave

protection both *in vitro* and in soil. The only difference between that result and our present study is the effect of UCMB-5033, but that difference indicates a high level of specificity in the plant-pathogen-beneficial bacteria interaction. Here we also found that *Bacillus* UCMB-5113 gave good protection against the bacterial pathogen *P. syringae*. UCMB-5036 gave an insignificant protection and the other strains provided no protection.

This study showed that *Bacillus*-mediated defense against one pathogen, does not necessarily also mediate a significant defense against another pathogen, having a different virulence mechanism. Known genomes of the *B. amyloliquefaciens* strains, the pathogen *P. syringae* as well as of *A. thaliana* give an opportunity to study colonisation and disease suppression at genetic and molecular levels.

4.3.2 Effects of *Bacillus* Strains on Plant Protection against Herbivory

Bacillus-treated *Arabidopsis* plants were also tested for any effects against the glucosinolate specialist insect pest *P. xylostella*, but no such effect was observed. To understand how the different protection effects are mediated and which primary signals that are involved will be investigated by the use of signaling mutants.

4.4 Mechanisms of *Bacillus* Priming

The event of priming in plants through beneficial bacteria has been studied most extensively with PGPR. These non-pathogenic rhizobacteria are known to colonies the plant root surface, and are capable of reducing disease incidence in above ground plant tissues through ISR (Pieterse et al., 1998; Knoester et al., 1999; van Wees et al., 2000). To further understand the mechanistic role of UCMB5113, we used *A. thaliana* for studies of induction of systemic resistance. *Bacillus* UCMB5113 applied to the roots of *Arabidopsis* Col-0 gave lower disease severity and pathogen proliferation in the leaves from hemibiotroph *Pst*DC3000 and nectrotroph *A. brassicicola* inoculations. UCMB5113 seems to be an ISR-inducing *Bacillus* strain, since it induces systemic resistance to a hemibiotroph and a nectrotroph, which is logic when compared to other reports about non-pathogenic bacteria colonizing *Arabidopsis* (Pieterse et al., 2002; Ton et al., 2002; Rudrappa et al., 2008; Niu et al., 2011).

4.4.1 Hormones Involved in *Bacillus* Priming

Earlier studies have reported that *A. brassicicola* infection can be controlled by enhanced JA signaling pathways (van Wees et al., 2003). The observations in

our study reveal that UCMB5113-treatment can elicit both SA and JA responses in the plant, depending on the pathogen challenge of the systemic tissues. The effect given by UCMB5113-treatment seem to be able to act on both JA and SA pathways depending on the type of pathogen, and a recent study by Niu et al. (2011) reported a simultaneous activation of SA and JA/ET signaling pathways in *Arabidopsis* on treatment with *B. cereus.*

In *A. thaliana*, ISR triggered by *P. fluorescens* is regulated by JA and ET dependent signaling pathways (Pieterse et al., 1998). Recent studies have shown that root colonization by *Bacillus subtilis* FB17 can restrict *Pst*DC3000 infection in the aerial plant parts by stomata closure through ABA and SA signaling (Kumar et al., 2012). Congruent with the study by Niu et al., (2011), our results also showed that *B. amyloliquefaciens* UCMB5113 is capable of activating both JA and SA, pathway depending on the type of pathogen.

4.4.2 Genes Involved in *Bacillus* Priming

Our studies suggest that UCMB5113 mediated ISR to *Pst*DC3000 in *Arabidopsis* occurs through the activation of SA via *NPR1, e*ven though we found a partial protection to *Pst*DC3000 in *Nah*G plants pre-treated with UCMB5113.

JAZ is a negative regulator of JA signaling, and is known to inhibit the expression of MYC2 transcription factor responsible for the activation of JA responsive genes. In this study, we saw attenuated *JAZ1* transcripts compared to the non-treated plants as a result of UCMB5113 treatment followed by inoculation with *P. syringae* (DC3000), but at the same time MYC2 transcripts were found to be up-regulated and at later time points the expression of *JAZ1* was subdued. This indicates that UCMB5113 could help the plant to repress the JAZ activation by simultaneously activating SA responsive signals.

The observed action of pathogenic bacterium *Pst*DC3000, uses a JA-Ile mimic signal Coronatine (COR) to activate *JAZ* genes during infection (Demianski et al., 2011). In our studies it seems that *Bacillus* modulate the activation of JA signaling through the repression of *JAZ1* genes, and stimulate SA responsive genes. *NPR1* seems to have an important role in deciding the defense response activation by *Bacillus* since UCMB5113 pre-treatment of *npr1-1* mutant plants did not give protection against growth of *Pst*DC3000, and this is similar to the results of previous studies (Pozo et al., 2008; Niu et al., 2011).

Necrotrophic fungi are known to survive in the host by killing the cells and feeding on the remains. Plants evade the pathogen onslaught by an alternative defense pathway mediated by JA (Glazebrook et al., 2003; Spoel et al., 2007). JA signaling is known to enhance levels of marker genes like *PDF1.2* and

VSP2 (Glazebrook, 2005). UCMB5113 pre-treatment seems to drive the expression of MYC2 upon challenge with *A. brassicicola*, a necrotrophic pathogen that can be restricted by enhanced JA levels. *VSP2* and *PDF1.2*, known JA markers, were found to be up-regulated upon UCMB5113-treatment and pathogen challenge. Elevated JA and JA-Ile levels in the UCMB5113 treated plants challenged with *A. brassicicola*, compared to the non-treated pathogen-challenged plants.

UCMB5113 mediated ISR against *A. brassicicola* was abolished in the *npr1-1* mutants and only a partial ISR was observed in the *jar1-1*, *coi1-1* and *Nah*G plants. These data implicate the prominent role of *NPR1* in *Bacillus* mediated priming in support with published data for other interactions (Rudrappa et al., 2008; Kawamura et al., 2009).

JAZ family members have been previously shown to have a differential response to *Pst*DC3000 stimuli (Demianski et al., 2011). Our results showed that *JAZ1* transcripts were suppressed by UCBM5113-treatment followed by inoculation with *Pst*DC3000; this gave us an opportunity to elucidate the role of JAZ1 in priming. UCMB5113 only partially restricted the growth of *Pst*DC3000 in *jaz1-1* mutant compared to the wild type Col-0, whereas, *myc2-2* mutants completely failed to activate ISR in response to UCMB5113. *MYC2* has also been previously reported to play a key role in beneficial bacteria priming (Pozo et al., 2008).

Previously, it has been shown that colonization of beneficial rhizobacteria like *P. fluorescens* leads to the expression of *MYB72* transcription factor in the roots of *A. thaliana* (van der Ent et al., 2008). In our study, we also observed an elevated expression of MYB72 in the roots of *Arabidopsis* upon colonization with *B. amyloliquefaciens* UCMB5113, and the *myb72-1* mutant failed to express ISR.

In our study we tried to elucidate the link between the MYB72 and MYC2. *myb72-1* mutants treated with UCMB5113, were not compromised in the expression of *MYC2* compared to the wild type Col-0. These results indicate the presence of other unidentified transcription factors that might be involved in the induction of *MYC2* upon treatment with UCMB5113.

It has been previously reported that during beneficial bacteria colonization of the root surface, MAMPs attenuate defense responses in the plants (Millet et al., 2010). However, recently it has been demonstrated in *Arabidopsis* during the initial stages of colonization, *Bacillus subtilis* suppress MAMPs elicited defense responses and rather triggers the recruitment of beneficial bacteria through the activation of root *ALMT1* expression (Lakshmanan et al., 2012).

Studies using semi-quantitative RT-PCR by Niu et al. (2011), has shown that root application of *B. cereus* on *Arabidopsis* resulted in an elevated expression of *PR1, PR2, PR5* and *PDF1.2* in the leaves but not in the roots.

In the present study, root treatment of UCMB5113 gave a gradual increase in the expression of *VSP2:GUS* both in the roots and in the leaves of *A. thaliana*, however the expression of *PDF1.2:GUS* was found to be down-regulated at an earlier time interval in the leaves of UCMB5113-treated plants compared to the water control. *PR1:GUS* expression was found only in the leaves, of the UCMB5113-treated plants.

The expression of starch biosynthetic genes in the primed plants indicates another role of *Bacillus* mediated resource allocation.

4.5 Overall Conclusions

We have demonstrated that beneficial bacteria of the species *B. amyloliquefaciens* can boost defense in oilseed rape against the generalist herbivore *S. littoralis*. The mechanism behind this effect seems to be due to JA-regulated priming, which was shown to have effects on primary metabolism and some effects on secondary metabolism. The given defense strategies where changed metabolism and resource allocation upon priming, and less of the more costly direct defenses.

Increasing concerns in environmental issues gives microbial biocontrol an exciting perspective, as the use of naturally occurring soil microbes instead of harmful chemicals provides a very promising alternative for crop protection. The mechanisms of PGPB plant colonization and improved growth and stress tolerance are not well known (Compant et al., 2010). The *Bacillus* genus is ubiquitous in nature and houses a variety of known species that all can produce endospores. There is support for compatibility of *Bacillus* in agricultural systems (McSpadden Gardener, 2004), although specific strains have not been evaluated thoroughly with for applied work.

Another interesting issue is the *Bacillus* colonization. Soil is a very complex system to analyze (Lombard et al., 2011), but with the here described qPCR assays, we are now able to study some of the steps in plant-*Bacillus* interactions in the rhizosphere that finally leads to root colonization and plant protection. Of high interest is the role of plant genotype and root exudates for recruitment of *Bacillus* bacteria (Rudrappa et al., 2008). Use of *Bacillus* bacteria to boost crop production, means that effects on ecosystems must be elucidated.

An additional conclusion of these studies is that *Bacillus* mediated protection is provided by the same strains in *Arabidopsis* as in *B. napus,* with

the exception of strain UCMB-5033. Establishment of *Arabidopsis* as model system for *Bacillus* interactions enables mechanistic studies of colonization, growth promotion and stress protection.

References

Agarwal, P.K., Agarwal, P., Reddy, M. K and Sopory, S.K. (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports*, vol. 25, pp. 1263-1274.

Ahmad, S., van Hulten, M., Martin, J., Pieterse, C.M.J., van Wees, S.C.M. and Ton, J. (2011). Genetic dissection of basal defense responsiveness in accessions of *Arabidopsis thaliana*. *Plant, Cell & Environment*, vol. 34 (7), pp. 1191-1206.

Ahmed, M.S., Sallam, N.M.A., Mohamed A.A. and Hassan Mohamed H.A. (2013). Effect of mycorrhiza and biofertilisers on reducing the incidence of *Fusarium* root and pod rot diseases of peanut. *Archives of Phytopathology and Plant Protection*, vol. 46, (7), pp. 868-881.

Ahn, I-P., Lee, S-W. and Suh, S-C. (2007). Rhizobacteria-induced priming in *Arabidopsis* is dependent on ethylene, jasmonic acid, and NPR1. *Molecular Plant-Microbe Interactions*, vol. 20 (7), pp. 759–768.

Arimura, G-I., Ozawa, R. and Maffei, M.E. (2011). Recent advances in plant early signaling in response to herbivory. International Journal of Molecular Sciences, vol. 12, pp. 3723-3739.

Bagchi, S., Namgail, T. and Ritchie, M.E. (2006). Small mammalian herbivores as mediators of plant community dynamics in the high-altitude arid rangelands of Trans-Himalaya. *Biological Conservation*, vol. 124 (4), pp. 438-442.

Bais, H.P., Weir, T.L., Perry, LG., Gilroy, S. and Vivancol, J.M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual. Review in Plant Biology*, vol. 57, pp. 233‑66.

Baker, N.R. and Rosenqvist, E. (2004). Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany*, vol. 55 (403), pp. 1607-1621.

Balachandran S., Hurry V.M., Kelley S.E., Osmond C. B., Robinson S.A., Rohozinski J., Seaton, G.G.R. and Sims, D.A. (1997). Concepts of plant biotic stress. Some insights into the stress physiology of virus-infected plants, from the perspective of photosynthesis. *Physiologia Plantarum*, vol. 100, pp. 203-213.

Barea, J-M., Azcón, R. and Azcón-Aguilar, C. (2002). Mycorrhizosphere interactions to improve plant fitness and soil quality. *Antonie van Leeuwenhoek*, vol. 81 (1-4), pp. 343-351.

Bejai, S.R., Danielsson, J. and Meijer, J. (2009). Transcript profiling of oilseed rape (*Brassica napus*) primed for biocontrol differentiate genes involved in microbial interactions with beneficial *Bacillus amyloliquefaciens* from pathogenic *Botrytis cinerea*. *Plant Molecular Biology*, vol. 70, (1-2), pp. 31-45.

Bejai, S., Fridborg, I. and Ekbom, B. (2012). Varied response of *Spodoptera littoralis* against *Arabidopsis thaliana* with metabolically engineered glucosinolate profiles. *Plant Physiology and Biochemistry*, vol. 50, pp. 72–78.

Bhattacharyya, P.N and Jha, D.K (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, vol. 28 (4), pp. 1327-1350.

Bodnaryk, R.P. (1994). Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry*, vol 35 (2), pp. 301-305.

Bouwmeester, H.J, Matusova, R., Zhongkui, S. and Beale, M.H. (2003). Secondary metabolite signaling in host–parasitic plant interactions. *Current Opinion in Plant Biology*, vol. 6 (4), pp. 358-364.

Brodersen, P., Petersen, M., Nielsen, H.B., Zhu, S., Newman, M-A., Shokat, K.M., Rietz, S., Parker, J. and Mundy, J. (2006). *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *The Plant Journal*, vol. 47, (4), pp. 532–546.

Bruessow, F., Gouhiel-Darimont, C., Buchala, A., Metraux, J-P. and Reymond, P. (2010). Insect eggs suppress plant defense against chewing herbivores. *The Plant Journal*, vol. 62 (5), pp. 876-885.

Bryant, J.P., Provenza, F.D, Pastor, J., Reichardt, P.B., Clausen, T.P. and du Toit, J.T. (1991). Interactions between woody plants and browsing mammals mediated by secondary metabolites. Annual Review of Ecology and Systematics, vol. 22, pp. 431-446.

Casati, P., Drincovich, M.F., Edwards, G.E. and Andreo, C.S. (1999). Malate metabolism by NADP-malic enzyme in plant defense. *Photosynthesis Research*, vol. 61, pp. 99-105.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, .B, Jungblut, P.R, Vater, J., Süssmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G. and Borriss, R. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nature Biotechnology*, vol. 25 (9), pp. 1007-1014.

Chen, M-S. (2008). Inducible direct plant defense against insect herbivores: A review. *Insect Science*, vol 15 (2), pp. 101–114.

Chen, X.H, Koumoutsi, A., Scholtz, R., Vater, J., Süssmuth, R. and Borriss, P.R. (2009). Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *Journal of Biotechnology*, vol. 140 (1–2), pp. 27–37.

Chet, I., Viterbo, A., Brotman, Y. and Lousky, T. (2006). Enhancement of plant disease resistance by the biocontrol agent *Trichoderma*. Life Science Open Day | 2006 | Weizmann Institute of Science, www.weizmann.ac.il/Biological_Chemistry/scientist/Chet/Chet.html.

Chico, J.M., Chini, A., Fonesca, S. and Solano, R. (2008). JAZ repressors set the rhythm in jasmonate signaling. *Current Opinion in Plant Biology*, vol. 11, pp. 486-494.

Cho, S.M., Kang, E.Y., Kim, M.S., Yoo, S.J., Im, Y.J., Kim, Y.C., Yang, K.Y., Kim, K.Y. Kim, K.S., Choi, Y.S., Chob, B.H. (2010). Jasmonate-dependent expression of a galactinol synthase gene is involved in priming of systemic fungal resistance in *Arabidopsis thaliana*. *Botany*, vol. 88(5), pp. 452-461.

Chung, H.S., Koo, A.J.K., Gao, X., Jayanty, S., Thines, B., Howe, G.A. (2008), Regulation and function of *Arabidopsis* JASMONATE ZIM-domain genes in response to wounding and herbivory. *Plant Physiology*, vol. 146, pp. 952-964

Chung, H.S., Niub, Y., Browseb, J., Howe, G.A. (2009). Top hits in contemporary JAZ: An update on jasmonate signaling. *Phytochemistry*, volume 70 (13–14), pp. 1547–1559.

Compant, S., Clement, C. and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, vol. 42, pp. 669-678.

Conrath, U., Beckers, G.J.M, Flors, V., García-Augustin, P., Jakab, G., Mauch, F., Newman, M-A., Pieterse, C.M.J, Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. and Mauch-Mani, B. (2006). Priming: Getting Ready for Battle. *Molecular Plant-Microbe Interactions*, vol. 19 (10), pp. 1062-1071.

Conrath, U. (2011). Molecular aspects of defense priming. *Trends in Plant Science*, vol. 16 (10), pp. 524-531.

Cui, J., Bahrami, A.K, Pringle, E.G., Hernandez-Guzman, G., Bender, C.L.; Pierce, N.E. and Ausubel, F.M. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences USA*, vol. 102 (5), pp. 1791-1796.

Dangl, J.L. and Jones, J.D.G. (2001). Plant pathogens and integrated defense responses to infection. *Nature*, vol. 411, pp. 826-833.

Danielsson, J., Reva, O. and Meijer, J. (2007). Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. *Microbial Ecology*, vol. 54 (1), pp. 134-140.

Demianski, A.J., Chung, K.M. and Kunkel, B.N. (2011). Analysis of *Arabidopsis JAZ* gene expression during *Pseudomonas syringae* pathogenesis. *Molecular Plant Pathology*, vol. 13, pp. 46–57.

Dinant, S. and Lemoine, R. (2010). The phloem pathway: New issues and old debates. *Comptes Rendus Biologies*, vol. 333 (4), pp. 307-319.

de Weert, S., Vermeiren, H., Mulders, I.H., Kuiper, I. and Hendrickx, N. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions*,, vol. 15, pp. 1173-1180.

Doornbos, R.F., van Loon, L.C. and Bakker, P.A.H.M. (2012). Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agronomy for Sustainable. Development*, vol. 32, pp. 227–243.

Doughty, K.J, Kiddle, G.A., Pye, B.J., Wallsgrove, R.M. and Pickett, J.A. (1995). Selective induction of glucosinolates in oilseed rape leaves by methyl jasmonate. *Phytochemistry*, vol. 38 (2), pp. 347–350.

Durrant, W.E, Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology*, vol. 42, pp. 185-209.

Ehrenfeld, J.G., Ravit, B. and Elgersma, K. (2005). Feedback in the plant-soil system. *Annual Review of Environment and Resources*, vol. 30, pp. 75-115.

Erb, M., Meldau, S., Howe, G.A. (2012). Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science*, vol. 17 (5), pp. 250-259.

Farwell, A.J., Vesely, S., Nero, V., Rodriguez, H., McCormack, K., Shah, S., Dixon, D.G., Glick, B.R. (2007). Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site. *Environmental Pollution*, vol. 147 (3), pp. 540–545.

Fernandes, G.W. (1994). Plant mechanical defenses against insect herbivory. *Revista Brasileira de Entomologia*, vol. 38 (2), pp. 421-433.

Fridborg, I., Johansson, A., Lagensjö, J., Leelarasamee, N., Floková, K., Tarkowská, D., Meijer, J. and Bejai, S. (2013). ML3: a novel regulator of herbivory-induced responses in *Arabidopsis thaliana*. *Journal of Experimental. Botany*, 64 (4), pp. 935-948.

Fu, Z.Q., Dong, X. (2013). Systemic acquired resistance: Turning local infection into global defense. *Annual Review of Plant Pathology*, vol. 64, pp. xx-xx. DOI: 10.1146/annurev-arplant-042811-105606

Glazebrook, J., Chen, W., Estes, B., Chang, H-S., Nawrath, C., Metraux, J-P., Zhu, T., Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. The *Plant Journal*, vol. 31, pp. 217–228.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Reviews in Phytopathology*, vol. 43, pp. 205-227.

Gómez-Gómez, L., Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Molecular Cell*, vol., 5 (6), pp. 1003-1011.

Gutiérrez, S., Michalakis, Y., van Munster, M., Blanc, S. (2013). Plant feeding by insect vectors can affect life cycle, population genetics and evolution of plant viruses. *Functional Ecology*, vol. 27 (2), pp. 1-13. *Functional Ecology*. doi: 10.1111/1365-2435.12070

Hajra, N., Shahina F., and Firoza K. (2013). Biocontrol of root-knot nematode by arbuscular mycorrhizal fungi in Luffa cylindrica. Pakistan Journal of Nematology, vol. 31 (1), pp. 77-84.

Halkier, B.A. and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology*, vol. 57, pp. 303-333.

Hawes, M.C., Curlango-Rivera, G., Xiong, Z. and Kessler, J.O. (2012). Roles of root border cells in plant defense and regulation of rhizosphere microbial populations by extracellular DNA 'trapping'. *Plant Soil*, vol. 355, pp. 1-16.

Heil, M., Ibarra-Laclette, E., Adame-Álvarez, R.M., Martínez, O. and Ramirez-Chávez, E., Molina-Torres, J. and Herrera-Estrella, L. (2012). How plants sense wounds: damaged-self recognition is based on plant-derived elicitors and induces octadecanoid signaling. *PLoS ONE*, vol. 7(2), e30537.

Hilker, M., Kobs, C., Varama, M. and Schrank, K. (2001). Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *The Journal of Experimental Biology*, vol. 205, pp. 455–461.

Hind, S.R., Pulliam, S.E., Veronese, P., Shantharaj, A.N., Jacobs, N.S. and Stratmann, J.W. (2011). The COP9 signalosome controls jasmonic acid synthesis and plant responses to herbivory and pathogens. The Plant Journal, vol. 65 (3), pp. 480–491.

Hirsch, A.M. and Kapulnik, Y. (1998). Signal transduction pathways in mycorrhizal associations: Comparisons with the rhizobium–legume symbiosis. *Fungal Genetics and Biology*, vol. 23 (3), pp. 205–212.

Hirsch, A.M. (2004). Plant-microbe symbioses: A continuum from commensalism to parasitism. *Symbiosis*, vol. 37, pp. xx-xx. UC Los Angeles: Retrieved from: http://escholarship.org/uc/item/6kx779h1

Hodge, S., Pope, T.W., Holaschke, M., Powell, G. (2006). The effect of β-aminobutyric acid on the growth of herbivorous insects feeding on *Brassicaceae*. *Annals of Applied Biology*, vol. 148 (3), pp. 223-229.

Holopainen, J.K. and Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends in Plant Science*, vol 15 (3), pp. 176-184.

Ibraheem, O., Hove, R.M. and Bradley, G. (2008). Sucrose assimilation and the role of sucrose transporters in plant wound response. *African Journal of Biotechnology*, vol. 7, pp. 4850-4855.

Jung, H.W., Tschaplinski T.J., Wang, L., Glazbrook, J., Greenberg, J.T. (2009). Priming in systemic plant immunity. *Science*, vol. 324 (5923), pp. 89-91.

Karban, R. and Agrawal, A. (2002). Herbivor offense. *Annual Review of Ecology and Systematics*, vol. 33, pp. 641-664.

Katagiri, F., Thilmony, R. and He, S. (2002). The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *The Arabidopsis Book*, vol. 1, pp. 1-39, e0039

Katsir, L., Chung, H.S, Koo, A.J.K, Howe, G.A. (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. *Current Opinion in Plant Biology*, vol. 11 (4), pp. 428-435.

Kawamura, Y., Takenaka, S., Hase, S., Kubota, M., Ichinose, Y., Kanayama, Y., Nakaho, K., Klessing, D.F. and Takahashi, H. (2009). Enhanced defense responses in *Arabidopsis* induced by the cell wall protein fractions from *Pythium oligandrum* require SGT1, RAR1,NPR1 and JAR1. *Plant Cell Physiology*, vol. 50, pp. 924-934.

KEMI (2006) http://www.kemi.se/upload/trycksaker/pdf/statistik/forsalda_bkm_2005.pdf

Kessler, A., Halitschke, R., Diezel, C. and Baldwin, I.T. (2006). Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuate*. *Oecologia*, vol. 148 (2), pp. 280-292.

Kim, M.S., Cho, S.M, Kang, E.Y., Im, Y.J., Hwangbo, H., Kim, Y.C, Ryu, C-M., Yang, K.Y., Chung, G.C. and Cho, B.H. (2008). Jasmonate-dependent expression of a galactinol synthase gene is involved in priming of systemic fungal resistance in *Arabidopsis thaliana*. *Botany*, vol. 88 (5), 452-461.

Kloepper, J.W., Ryu, C-M. and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology*, vol. 94 (11), pp. 1259-1266.

Knoester, M., Pieterse, C.M.J., Bol, J.F. and van Loon, L.C. (1999). Systemic resistance in *Arabidopsis* induced by rhizobacteria re- quires ethylene-dependent signaling at the site of application. *Molecular Plant Microbe Interactions*, vol. 12, pp. 720–727.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., den Otter, F.C, van Loon, L.C. and Pieterse, C.M. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiology*, vol. 147 (3), pp. 1358-1368.

Koornneef, A. and Pieterse, C.M.J. (2008). Cross talk in defense signaling. *Plant Physiology*, vol. 146 (3), pp. 839-844.

Kumar, A.S., Lakshmanan, V., Caplan, J.L., Powell, D., Czymmek, K.J., Levia, D.F. and Bais, H.P. (2012). Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. *The Plant Journal*, vol. 72, pp. 694–706.

Kunkel, B.N. and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, vol. 5, (4), pp. 325‑331.

Lakshmanan, V., Kitto, S.L., Caplan, J.L., Hsueh, Y-H., Kearns, D.B., Wu, Y-S. and Bais, H.P. (2012). Microbe-associated molecular patterns (MAMPs)-triggered root responses mediate beneficial rhizobacterial recruitment in *Arabidopsis*. *Plant Physiology*, vol. 160 (3), pp. 1642-1661.

Laluk, K. and Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? *The Arabidopsis Book*, vol. 8, pp. 1-34, e0136.

Leon-Reyes, A., Spoel, S.H., de Lange, E.S., Abe, H., Kobayashi, M., Tsuda, S., Millenaar, F.F., Welschen, R.A.M, Ritsema, T. and Pieterse, C.M.J. (2009). Ethylene modulates the role of *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1* in cross talk between salicylate and jasmonate signaling. *Plant Physiology*, vol. 149 (4), pp. 1797-1809.

Lindermayr, C., Sell, S., Müller, B., Leister, D. and Durner, J. (2010). Redox Regulation of the NPR1-TGA1 System of *Arabidopsis thaliana* by Nitric Oxide. *The Plant Cell*, vol. 22 (8), pp. 2894-2907.

Livak, KJ. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, vol. 25 (4), pp. 402-408.

Lombard, N., Prestat, E., van Elsas, J.D. and Simonet, P. (2011). Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. *FEMS Microbiology Ecology*, vol. 78, pp. 31–49.

Manjula, K. and Podile, A.R. (2001). Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. *Canadian Journal of Microbiology*, 47, pp. 618–625.

Mauricio, R. and Rausher, M.D. (1997). Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution*, vol. 51 (5), pp. 1435-1444.

Maya, M.A. and Matsubara, Y. (2013). Tolerance to Fusarium wilt and anthracnose diseases and changes of antioxidative activity in mycorrhizal cyclamen. *Crop Protection*, vol. 47, pp. 41–48.

McSpadden Gardener, B.B. (2004). Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. *Phytopathology*, vol. 94, pp. 1252-1258.

Meyer, A., Grote, R. and Butterbach-Bahl, K. (2012). Integrating mycorrhiza in a complex model system: effects on ecosystem C and N fluxes. *European Journal of Forest Research*, vol. 131, pp. 1809-1831.

Millet, Y.A., Danna, C.H., Clay, N.K., Songnuan, W., Simon, M.D., Werck- Reichhart, D. and Ausubel, F.M.. (2010). Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell*, vol. 22, 973–990.

Moghaddam, M.R.B. and van den Ende, W. (2012). Sugars and plant innate immunity. *Journal of Experimental Botany*, vol. 63 (11), pp. 3989-3998.

Mosquera-Espinosa A.T., Bayman, P. Prado, G.A., Gómez-Carabalí, A. and Otero, J.T. (2013). The double life of *Ceratobasidium*: orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia solani* sheath blight of rice. *Mycologia*, vol. 105, pp. 141-150.

Mou, Z., Fan, W. and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell*, vol. 113, pp. 935–944.

Niu, D.D., Liu, H.X., Jiang, C.H., Wang, Y.P., Wang, Q.Y., Jin, H.L. and Guo, J.H.. (2011). The plant growth promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate and jasmonate/ethylene dependent signaling pathways. *Molecular Plant Microbe Interactions*, vol. 24, pp. 533-542.

O'Brien, J.A., Daudi, A., Butt, V.S. and Bolwell, G.P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*, vol. 236. pp. 765–779.

Orians, C.M. and Thorn, A. (2011). Herbivore-induced resource sequestration in plants: why bother? *Oecologia*, vol. 167, pp. 1–9.

Parker, C. (1991). Protection of crops against parasitic weeds. *Crop Protection*, vol. 10 (1), pp. 6-22.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., et al (2000). *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell*, vol. 103, pp. 1111–1120.

Pieterse, C.M.J., van Wees, S.C., Hoffland, E., van Pelt, J.A., van Loon, L.C., (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell*, vol. 8, pp. 1225-37.

Pieterse, C.M, van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *The Plant Cell*, vol. 10 (9), pp. 1571-1580.

Pieterse, C.M.J., Ton, J. and van Loon, L.C. (2001). Cross-talk between plant defence signalling pathways: boost or burden? *AgBiotechNet*, vol. 3, pp, 1-8.

Pieterse, C.M.J., van Wees, S.C.M., Ton, J., van Pelt, J.A. and van Loon, L.C. (2002). Signalling in Rhizobacteria-Induced Systemic Resistance in *Arabidopsis thaliana*. *Plant Biology*, vol. 4 (5), pp. 535–544.

Pineda, A., Zheng, S-J., van Loon, J.J.A, Pieterse, C.M.J. and Dicke, M. (2010). Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Science*, vol. 15 (9), pp. 507-514.

Porcel, R., Aroca, R. and Ruiz-Lozano, J.M. (2012). Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. *Agronomy for Sustainable Development*, vol. 32 (1), pp. 181-200.

Pozo, M.J., van der Ent, S., van Loon, L.C. and Pieterse, C.M.J. (2008). Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis* thaliana. *New Phytologist*, vol. 180 (2), pp. 511-523.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Molecular Evolution*, vol. 42, pp. 93-113.

Reva, O. N., Dixelius, C., Meijer, J., Priest, F.G. (2004). *FEMS Microbiology Ecology*, vol. 48 (2), pp. 249-259.

Rudrappa, T., Czymmek, K.J, Paré, P.W. and Bais, H.P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology*, vol. 148 (3), pp. 1547-1556.

Rudrappa, T., Biedrzycki, M.L, Kunjeti, S.G., Donofrio, N.M, Czymmek, K.J., Paré, P.W., Bais, H.P. (2010). The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. *Communicative & Integrative Biology*, vol. 3 (2), pp. 130 – 138.

Saharan, B.S. and Nehra, V. (2011). Plant growth promoting rhizobacteria: A critical review. Life Sciences and Medicine Research, vol. 2011, pp. 1-30.

Samuels, G.J. (1996). *Trichoderma*: a review of biology and systematics of the genus. *Mycological* Research, vol. 100 (8), pp. 923‑935.

Schlaeppi, K., Bodenhausen, N., Buchala, A., Mauch, F., Reymond, P. (2008). The glutathione-deficient mutant *pad2-1* accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant Journal*, vol. 55, pp. 774–786.

Schwachtje, J., Minchin, P.E.H, Jahnke, S., van Dongen, J.T, Schittko, U. and Baldwin, I.T. (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proceedings of the National Academy of Sciences USA*, vol. 103 (34), pp. 12935-12940.

Schwachtje, J., Karojet, S., Kunz, S., Brouwer, S. and van Dongen, J.T. (2012). Plant-growth promoting effect of newly isolated rhizobacteria varies between two *Arabidopsis* ecotypes. *Plant Signaling & Behavior*, vol. 7 (6), pp. 623-627.

Sharma, A. and Yadav, S. (2013). Review on role of VAM fungi in crop plant-soil system. *International Journal of Agricultural Science Research*, vol. 3 (1), pp. 17-24.

Sharma, P., Kumar P.V., Ramesh, R., Saravanan, K., Deep, S., Sharma, M., Mahesh, S. and Dinesh, S. (2011). Biocontrol genes from *Trichoderma* species: A review. *African Journal of Biotechnology*, vol. 10 (86), pp. 19898-19907.

Shimono, M., Sugano, S., Nakayama, A., Jiang, C-J., Ono, K., Toki, S. and Takatsuji, H. (2007). Rice WRKY45 Plays a Crucial Role in Benzothiadiazole-Inducible Blast Resistance. *The Plant Cell*, vol. 19 (6), pp. 2064-2076.

Si-Ammour, A., Mauch-Mani, B. and Mauch, F. (2003). Quantification of induced resistance against *Phytophthora* species expressing GFP as a vital marker: β-aminobutyric acid but not BTH protects potato and *Arabidopsis* from infection. *Molecular Plant Pathology*, vol. 4 (4), pp. 237–248.

Spanu, P. and Kämper, J. (2010). Genomics of biotrophy in fungi and oomycetes — emerging patterns. *Current Opinion in Plant Biology*, vol. 13 (4), pp. 409-414.

Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, A.J, Métraux, J-P., Brown, R., Kazan, K., van Loon, L.C., Dong, X. and Pieterse, C.M.J. (2003).

NPR1 Modulates Cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *The Plant Cell*, vol. 15 (3), pp. 760-770.

Spoel, S.H., Johnson, J.S. and Dong, X. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *PNAS*, vol. 104 (47), pp. 18842-18847.

Staswick, P.E. (2008). JAZing up jasmonate signaling. *Trends in Plant Science*, vol. 13 (2), pp. 66-71.

Stein, E., Molitor, A., Kogel, K.H., Waller, F. (2008). Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signalling and the cytoplasmic function of NPR1. *Plant and Cell Physiology*, vol. 49, pp. 1747–1751.

Tester, M. and Langridge, P. (2010). Breeding technologies to iIncrease crop production in a changing world. *Science*, vol. 327 (5967), pp. 818-822.

Thomma, B.P.H.J., Nelissen, I., Eggermont, K., Broekaert, W.F. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola. The Plant Journal,* vol. 19, pp. 163–171.

Tjamos, S.E., Flemetakis, E., Paplomatas, E. and Katinakis, P. (2005). Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Molecular Plant-Microbe Interactions*, vol. 18 (6), pp. 555-561.

Ton, J., van Pelt, J.A., van Loon ,L.C. and Pieterse, C.M.J. (2002). The *Arabidopsis* ISR1 locus is required for rhizobacteria-mediated induced systemic resistance against different pathogens. *Plant Biology*, vol. 4, pp. 224-227.

Ton, J., Jakab, G., Toquin, V., Flors, V., Iavicoli, A., Maeder, M., Métraux, J-P. and Mauch-Mania , B. (2005). Dissecting the β-aminobutyric acid–induced priming phenomenon in *Arabidopsis*. *the Plant Cell*, vol. 17 (3), pp. 987-999.

Tsai, C-H., Singh, P., Chen, C-W., Thomas, J., Weber., J., Mauch-Mani, B. and Zimmerli, L. (2011). Priming for enhanced defence responses by specific inhibition of the *Arabidopsis* response to coronatine. *The Plant Journal*, vol. 65 (3), pp. 469-479.

Valluru, R. and Van den Ende, W. (2011). Myo-inositol and beyond - Emerging networks under stress. *Plant Science*, vol. 181, pp. 387-400.

van der Ent, S., Verhagen, B.W., van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J., Joosten, R.G., Proveniers, M.C., van Loon, L.C., Ton, J. and Pieterse, C.M.J. (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiology*, vol. 146, pp. 1293-1304.

van Hulten, Marieke, Pelser, M., van Loon, L.C, Pieter, C.M.J. and Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA*, vol. 103 (14), pp. 5602-5607.

van Loon, J.J.A, de Boer, J.G and Dicke, M. (2000). Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomologia Experimentalis et Applicata*, vol. 97 (2), pp. 219-227.

van Oosten, V.R, Bodenhausen, N., Reymond, P., van Pelt, J.A, van Loon, L.C., Dicke, M. and Pieterse, C.M.J. (2008). Differential effectiveness of microbially induced resistance against herbivorous insects in *Arabidopsis*. *Molecular Plant-Microbe Interactions*, vol. 21 (7), pp. 919-930.

van Wees, S.C.M., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M.J. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences USA*, vol. 97, pp. 8711-8716.

van Wees, S.C.M., Chang, H.S., Zhu, T. and Glazebrook, J. (2003). Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiology*, vol. 132, pp. 606–617.

Verhage, A., Vlaardingerbroek, I., Raaijmakers, C., Dam, N.M., van Dicke, M., van Wees, S.C.M. and Pieterse, C.M.J. (2011). Rewiring of the jasmonate signaling pathway in *Arabidopsis* during insect herbivory. *Frontiers in Plant Science*, vol. 2, pp. 1–12.

Verhagen, B.W.M., Glazebrook, J., Zhu, T., Chang, H-S., van Loon, L.C. and Pieterse, C.M.J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Molecular Plant–Microbe Interactions*, vol. 17, pp. 895–908.

Verma, M., Brar, S.K., Surampalli, R.Y. and Valéro, J.R. (2007). Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochemical Enginering Journal*, vol. 37 (1), pp. 1-20.

Vos, C., Schouteden, N., van Tuinen, D., Chatagnier, O., Elsen, A., De Waele, D., Panis, B. and Gianinazzi-Pearson, V. (2013). Mycorrhiza-induced resistance against the root–knot nematode *Meloidogyne incognita* involves priming of defense gene responses in tomato. *Soil Biology and Biochemistry*, vol 60, pp. 45–54.

Walker, T.S, Bais, H.P., Grotewold, E. and Vivanco, J.M. (2003). Root exudation and rhizosphere biology. *Plant Physiology*, vol. 132 (1), pp. 44-51.

Wang, Z., Mao, H., Dong, C., Ji, R., Cai, L., Fu, H. and Liu, S. (2009). Overexpression of *Brassica napus MPK4* enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape. *Molecular Plant-Microbe Interactions*, vol. 22, pp. 235-244.

Ward J., Harris C., Lewis J., Beale M. (2003). Assessment of 1H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry*, vol. 62, pp. 949–957.

Ward, J.L., Forcat, S., Beckmann, M., Bennett, M., Miller, S.J., Baker, J.M., Hawkins, N.D., Vermeer, C.P., Lu, C., Lin, W., Truman, W.M., Beale, M.H., Draper, J., Mansfield, J.W. and Grant, M. (2010). The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Plant Journal*, vol. 63, pp. 443–457.

Weller, D.M, Mavrodi, D.V., van Pelt, J.A., Pieterse, C.M.J., van Loon, L.C. and Bakker, P.A.H.M. (2011). Induced systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* by 2,4-Diacetylphloroglucinol-producing *Pseudomonas fluorescens*. *Phytopathology*, vol. 102 (4), pp. 403-412.

Widarto, H.T., van Der Meijden, E., Lefeber, A.W.M., Erkelens, C., Kim, H.K., et al., (2006) Metabolomic differentiation of *Brassica rapa* following herbivory by different insect instars using two-dimensional nuclear magnetic resonance spectroscopy. *Journal of Chemical Ecology*, vol. 32, pp. 2417-2428.

Wittstock, U., Agerbirk, N., Stauber, E.J., Olsen, C.E., Hippler, M., Mitchell-Olds, T., Gershenzon, J. and Vogel, H. (2004). Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences USA*, vol. 101 (14), pp. 4859-4864.

Wu, J. and Baldwin, I.T. (2010). New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics,* vol. 44, pp. 1-24.

Wu, Y., Zhang, D.; Chu, J.Y, Boyle, P., Wang, Y., Brindle, I.D, De Luca, V. and Després, C. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports*, vol. 1, pp. 639–647.

Yang, J., Kloepper, J.W., and Ryu, C-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science*, vol. 14, (1), pp. 1-4.

Zamioudis, C. and Pieterse, C.M.J. (2012). Modulation of host immunity by beneficial microbes. *Molecular Plant Microbe Interactions*, vol. 25, pp. 139-150.

Zimmerli, L., Gabor, J., Métraux, J-P. and Mauch-Mani, B. (2000). Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β-aminobutyric acid. *PNAS*, vol. 97 (23), pp. 12920-12925.

Zimmerli, L., Métraux, J.P. and Mauch-Mani, B. (2001). β-Aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea. Plant Physiol*, vol. 126, pp. 517-523.

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