Tripartite interactions between *Brassica oleracea*, soil microbes, and shoot- and root feeding insects

Julia Friman

Propositions

1. Recruitment of plant rhizosphere microbes in response to aboveground herbivory is a type of indirect plant defense. (this thesis)

2. The protective effect from rhizobacteria on plants depends on the attacking insect species. (this thesis)

3. Independent researchers do not exist.

4. Scientific referencing resembles casting votes at the Eurovision song contest.

5. The societal benefit of research is not a matter of costs and benefits.

6. The Corona pandemic shows that we underestimate the ability of older people to adapt to new digital and societal solutions.

Propositions belonging to the thesis entitled:

"Tripartite interactions between *Brassica oleracea*, soil microbes and shoot- and root feeding insects"

Julia Friman, Wageningen, 28 June 2021

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Julia Friman

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences

Tripartite interactions between *Brassica oleracea*, soil microbes and shoot- and root feeding insects

Julia Friman

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 28 June 2021
at 11 a.m. in the Aula.

Julia Friman
Tripartite interactions between *Brassica oleracea*, soil microbes and shoot- and root feeding insects,
168 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2021)
With references, with summaries in English, Dutch and Swedish

ISBN: 978-94-6395-802-8
DOI: https://doi.org/10.18174/546148

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Chapter 1

General introduction

Cultivar	A cultivated plant variety
Endophyte	A non-pathogenic organism living inside a plant
Epiphyte	A non-pathogenic organism living on the surface of a plant
Exudate	Secreted substance
Genotype	An individual's genetic make-up
Gnotobiotic	In a situation where only known microbes are present
ISR	Induced Systemic Resistance resulting from interaction of a plant's roots with non-pathogenic rhizobacteria
Pathogen	A disease causing organism
PGPR	Plant growth promoting rhizobacteria
Phenotype	The set of characteristics of an individual caused by its genotype in interaction with environmental effects
PSF	Plant-soil feedback
Rhizo-	Root-associated
Rhizosphere	A narrow zone around the root that is influenced by root exudates
Siderophore	A small molecule with high iron affinity, used to transport iron into the organism
Variety	A group selected from a species, distinguished by common characteristics

Plants interact with a vast number of species (Schoonhoven *et al.*, 2005; Stam *et al.*, 2014), and through growth, they can modify their environment and influence the plant-associated species. Along the plant root alone, plants interact with and modify composition of tens of thousands of soil microbial species (Berg & Smalla, 2009; Yuan *et al.*, 2015a). This complex microbial community is crucial for plant health (Berendsen *et al.*, 2012). Furthermore, specific soil bacteria inhabiting the rhizosphere can colonize the plant root (Muci *et al.*, 2012; Pieterse *et al.*, 2014; Yuan *et al.*, 2015b). These so-called rhizobacteria may benefit the plant as symbionts in several ways. In sufficient densities, the root bacteria can stimulate plant growth (Pieterse *et al.*, 2012) through mechanisms such as improving plant nutrient access and uptake (Hayat *et al.*, 2010). The rhizobacteria can also influence the plant defense strategies such as resistance and tolerance, preparing the plant's defensive system for future attacks (Pieterse *et al.*, 2014).

Rhizobacteria and systemic resistance against insects

Plant defense consists of constitutive and induced defenses. The induced defenses are regulated through a complex phytohormonal network. While many phytohormones are involved in plant defense, there are three main phytohormonal regulators: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). These compounds and their derivatives are upregulated during an attack by insects or pathogens, which leads to increased production of defensive compounds as well as defensive

structures (Howe & Jander, 2008; Kariyat *et al.*, 2017). The phytohormones are upregulated differently depending on how the insect attacks the plant. Jasmonic acid production, often associated with ethylene, is upregulated when the plant is attacked by an insect that chews on the plant. Insects that instead feed from the plant's sap streams through a stylet, generally upregulate salicylic acid (Erb *et al.*, 2012b).

Plant defense can be primed against a broad range of attackers by some specific rhizobacteria (Pieterse *et al.*, 2014; Tsukanova *et al.*, 2017). This priming, termed induced systemic resistance (ISR), is a state of 'preparedness', and can be compared to an insurance for the plant. The priming is hypothesized to cost the plant some initial resources, but will enable quicker upregulation of defense and thereby reduce the negative consequences of an attack (Conrath *et al.*, 2006; Conrath, 2011; Martinez-Medina *et al.*, 2016). Interestingly, the rhizobacterially associated priming may last over plant generations through epigenetic changes in the plant genome (Pastor *et al.*, 2013).

Rhizobacterially primed ISR was shown to be effective against many pathogens and some insects *via* plant-mediated responses (Raaijmakers *et al.*, 2009; Pineda *et al.*, 2010; Rybakova *et al.*, 2017). However, previous research shows that rhizobacterial colonization and priming does not always work to the plant's advantage. For chewing insects, the rhizobacterial colonization can have a negative effect on the insect *via* plant-mediated effects (Badri *et al.*, 2013), yet there are examples of positive effects on insect performance as well (Dean *et al.*, 2014). Meanwhile, insects that feed on the plant's phloem may benefit from by rhizobacterial colonization. Some studies indicate an improved performance of piercing-sucking insects, when feeding on rhizobacterially colonized plants (Pineda *et al.*, 2012; Shavit *et al.*, 2013). The existing literature mainly focuses on rhizobacterial plant growth promotion. In contrast, less is known of the rhizobacterial effect on insect herbivores (Pineda *et al.*, 2010).

Rhizobacteria in agriculture

The effectiveness of employing rhizobacteria in agriculture as stimulators for plant growth and plant defense depends on successful bacterial root colonization, where dissemination is an important part. How and when to add the microbes to the crop's roots, to steer the microbiome to obtain a higher yield, are still not fully resolved. After a one-time application of a bacterial suspension alone, the number of rhizobacteria may rapidly decrease, possibly because the introduced bacteria have to compete with the native bacteria present in the soil (Bashan *et al.*, 2014). An application like this may give unpredictable results in terms of yield.

By utilizing a carrier, the bacteria may have shelter and protection (Taylor *et al.*, 1998; Wright *et al.*, 2017; Rocha *et al.*, 2019). The carrier can be a seed coat, granules or similar. The carrier could also be the seed itself. Application of bacteria directly on the seed is possible through a method called seed bio-priming (O'Callaghan, 2016). The method is relatively inexpensive compared to other methods such as seed coating, and can be done by the grower in-house. Seed bio-priming has shown to promote plant growth (Abuamsha *et al.*, 2011b). Yet, this method has rarely been investigated in terms of plant resistance against insects.

Bacterial communities over time

A plant can modify the soil microbial species composition through growth, and these modifications can also affect plants growing subsequently in the same soil (van der Putten *et al.*, 2013; Lekberg *et al.*, 2018). This 'legacy' effect on plants is a mechanism termed plant-soil feedback (PSF). Plant-soil feedbacks originating from plants acting upon a plant of the same species is referred to as conspecific. A positive plant-soil feedback improves the performance of conspecifics by making the soil environment more suitable, whereas a negative feedback lowers the suitability of the soil environment to conspecific plants.

Plants that are under attack by insect herbivores modify their soil microbiome composition, which can affect future plants growing in the same soil, which in turn can affect future herbivores feeding on these future plants (Kostenko *et al.*, 2012). As these feedbacks are mediated through the soil microbiome (Hu *et al.*, 2018; Bennett & Klironomos, 2019; De Long *et al.*, 2019), addition of a rhizobacterium could influence a future generation plant's growth and defense. Yet, knowledge is lacking on PSF effects coming from attack by herbivorous insects on plants, and how these interactions can be modified by rhizobacterial addition.

Main objective and research questions

The main objective of this thesis is to increase our understanding of plant growth and defense *via* rhizobacterial modifications. I focus on plant resistance against insects during different types of rhizobacterial inoculations by asking the following questions:

1. What are the effects of rhizobacteria on the performance of *Brassica*-associated herbivorous insects, and to what extent can these effects be explained by gene expression of defense signaling pathways and leaf chemistry?

2. How does applying beneficial rhizobacteria according to the seed bio-priming method lead to suppression of herbivore damage and promotion of plant growth compared to adding bacteria to the soil?
3. What are the effects of adding rhizobacteria on plant resistance when both an aboveground and belowground insect herbivore are feeding simultaneously on cabbage plants?
4. How does plant-soil-feedback affect the performance of a belowground insect herbivore, when soil is preconditioned with plants attacked by *Brassica*-associated herbivorous insects?

Study system

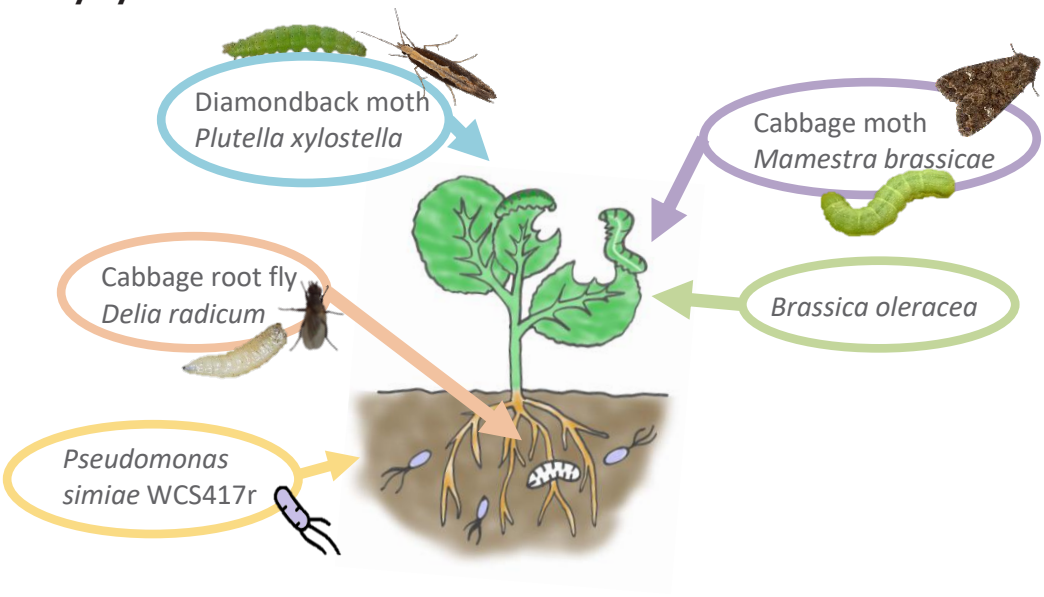

Figure 1 Model system used in this thesis.

To study the plant-mediated interactions between rhizobacteria and insect herbivores using different rhizobacterial inoculation methods, I use *Brassica oleracea* variety *gemmifera* cultivar Cyrus and variety *capitata* cultivar Christmas Drumhead (Fig. **1**). Cultivated *Brassica oleracea* descends from wild cabbage native to western Europe. The genus *Brassica* is extensively used in agriculture and is well-known in the literature, with knowledge ranging from ecology to genetics and from fundamental to applied aspects. Nevertheless, the effect of rhizobacteria on cabbage growth and the associated insect herbivore response has not yet been explored.

Among the most important *B. oleracea* pest herbivores are the leaf chewers Diamondback moth, *Plutella xylostella* L. (Lepidoptera:

Plutellidae), the Cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and the root chewer Cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae). *Plutella xylostella* is a specialist herbivore on brassicaceous plants (Talekar & Shelton, 1993; Furlong *et al.*, 2013). The lifecycle is around two weeks at 20°C, with four larval instars where the first larval instar is a leaf miner. The larvae can wriggle intensely when disturbed, and spin silk that attaches the larvae to the leaf, and which can be used to escape predators. The adult moth is colored grey and brown with a diamond-shaped pattern on the wings. The adult can migrate long distances. Continuous use of insecticide in agriculture has increased the resistance of the species towards pesticides, resulting in higher demands from agriculture to other pest control measures (Furlong *et al.*, 2013). *Mamestra brassicae* is a generalist herbivore feeding from at least 70 plant species. The insect has six larval instars, with a four-week life cycle at 20°C. The larvae have a light stripe along the body, with a dark green dorsal region, while the adult moths have speckled grey wings. *Delia radicum* has been considered a pest insect for more than 180 years (Schoene, 1916). Female flies lay eggs on the soil near the stem of the plant, and the larvae then mine into the roots. After around six weeks at 20°C they emerge as flies. There are no reports on the effect of any rhizobacteria on cabbage root fly to our knowledge.

The rhizobacterium *Pseudomonas fluorescens* WCS417r was renamed after sequencing to *P. simiae* WCS417r (Berendsen *et al.*, 2015). This epiphytic rhizobacterium was isolated from wheat (*Triticum aestivum* L.) in the Netherlands (Lamers *et al.*, 1988). The strain promotes *Arabidopsis thaliana* Heynh. (Brassicaceae) plant growth and defense (Pieterse *et al.*, 1996; Verhagen *et al.*, 2004; Zamioudis *et al.*, 2013). *Pseudomonas simiae* WCS417r does not produce the plant hormone auxin when grown on agar, but nevertheless stimulates *A. thaliana* lateral root development and root hair formation, and subsequently improves plant growth (Zamioudis *et al.*, 2013). *Pseudomonas simiae* WCS417r growth promotion and rhizobacterial priming are two different mechanisms in *Arabidopsis*. Inoculation with *P. simiae* WCS417r shows that *A. thaliana* growth promotion is independent from JA and ET signaling, compared to ISR, which is dependent on intact JA signaling (Zamioudis *et al.*, 2013). Interestingly, *P. simiae* WCS417r can produce SA when grown alone on agar (Ran *et al.*, 2005), but the significance of this feature in soil is not known (Bakker *et al.*, 2014). Previously work with *A. thaliana* and *P. simiae* WCS417r showed that some insects are affected by inoculation. Effects of inoculation of *A. thaliana* with *P. simiae* WCS417r on *Mamestra brassicae* ranged from negative (Pangesti *et al.*, 2017), positive (Pangesti *et al.*, 2015b; Fernández de Bobadilla *et al.*, 2017), and either negative

or positive depending on soil nutrient level (Pangesti *et al.*, 2015a). Effects of *P. simiae* WCS417r inoculation *via* plant-mediated effects on *P. xylostella* and *D. radicum* performance are as yet unknown.

Thesis outline

To study the plant-mediated interactions between rhizobacteria and insect herbivores during different rhizobacterial inoculation methods in-depth, I start this thesis with a literature exploration in Chapter 2. In the following experimental chapters, I narrow the focus to the plant-insect interactions between *Brassica oleracea* and various associated insect herbivores as discussed above, to investigate plant growth and insect resistance after rhizobacterial colonization.

Chapter 2. This chapter consists of a literature review of rhizobacterial effects on insect herbivores *via* plant-mediated effects. The chapter focusses on an update on the literature on rhizobacteria-plant-insect interactions. Further, I review bi-directional effects between rhizobacteria and insects *via* plant-mediated interactions.
Chapter 3. Chapter 3 focuses on plant growth and resistance in *B. oleracea* plants grown in *P. simiae* WCS417r-inoculated soil, against associated insect herbivores. In a greenhouse study, I mixed the bacteria into sterilized soil, sowed sterilized seeds and when grown, the plants were infested with either *D. radicum*, *P. xylostella* or *M. brassicae*. I measured plant and insect growth, phytohormonal levels, plant gene expression and plant growth, and C/N concentration in leaves.
Chapter 4. In this chapter, I added bacteria to the *B. oleracea* seeds according to the bio-priming method, instead of adding rhizobacteria to the soil prior to sowing. I then infested the grown plants with either *D. radicum*, *P. xylostella* or *M. brassicae* and measured plant and insect responses.
Chapter 5. Previous studies on insect herbivore performance after feeding on a plant with added rhizobacteria, focused on one insect species feeding on a plant. In chapter 5 I included two insect species feeding simultaneously, to explore rhizobacterial modifications of plant resistance under dual attack after rhizobacterial soil inoculation.

Chapter 6. This chapter explores plant resistance as influenced by plant-soil feedback. First, soil was conditioned with plants under attack from different herbivores both above- and belowground, and the root microbiome was investigated. The conditioned soil was then used to grow a second set of plants. These plants were infested with *D. radicum*. Plant resistance was studied through gene expression and insect performance.

Chapter 7. Experiments and results presented in this thesis are discussed here, by comparing and linking the outcomes of each experimental chapter. I discuss possible factors that influence rhizobacterial effects on plant defense know from literature, together with my own findings. I further identify strengths and weaknesses in this thesis research and suggest future directions in this field of research.

Acknowledgments

I thank Marcel Dicke and Joop van Loon for the helpful comments on an earlier version of this chapter.

Chapter 2

Insect – plant – microbe interactions: plant-mediated interactions between rhizobacteria and herbivorous insects

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Published in a revised version in Ecological Entomology

Abstract

Plants are members of complex communities. They are exposed to a wide diversity of insect herbivores that are in turn attacked by a wide diversity of carnivorous enemies. Constitutive and induced plant defenses prevent herbivore attack or reduce its intensity. Induced defense includes the attraction of predators or parasitoids to shoot-feeding herbivores, or entomopathogenic nematodes to root-feeding herbivores.

Microbes in the rhizosphere, especially plant-growth promoting rhizobacteria (PGPR), influence both direct and indirect induced defense of plants against insects. These PGPR are supported by plants through root exudates.

Insect-induced plant defense is regulated by hormonal signal-transduction pathways. Upon insect herbivory, plant hormone profiles and subsequently root exudate profiles are altered. Modified root exudation may influence the community of rhizobacteria that colonize plant root.

Recent studies show that upon aboveground attack by insect herbivores or plant pathogens, plants may recruit rhizobacteria that enhance plant defense against the attackers. We review the bidirectional nature of microbe-plant-insect interactions and focus on the effects of beneficial rhizobacteria *via* modification of plant defense traits on insects as well as the effects of plant defense against insects on the microbiome in the rhizosphere. Such knowledge will be instrumental for the development of sustainable crop protection strategies.

Keywords Insect-plant interactions, inducible defense, plant growth promoting rhizobacteria, PGPR, community ecology

Introduction

A central issue in ecology is to understand the mechanisms that shape the dynamics of communities. Plants, as the basis of food webs, are members of a diverse community that includes microbial and macrobial species. As a sessile organism, each plant is exposed to mobile individuals from hundreds of species above- and belowground, with the most speciose groups consisting of microbes and insects (van der Putten *et al.*, 2001; Stam *et al.*, 2014; Cordovez *et al.*, 2019). To prevent attack or limit the impact of being attacked, plants have evolved various defenses in an evolutionary arms race with their attackers. These defenses have far-reaching consequences for the plant-associated community (Ohgushi, 2005; Stam *et al.*, 2014).

Plant defense has long been considered a trait of plants exclusively. However, it has become clear that enemies of insect herbivores, such as carnivores, can be a component of plant defense as well (Price *et al.*, 1980) and that plants can actively recruit carnivores upon damage by herbivorous insects (Stam *et al.*, 2014; Turlings & Erb, 2018) (Fig. **1, 1**). Thus, as a result of defense induction, the modification of plant phenotype leads to changes in the plant-associated insect community, which has mostly been studied for aboveground communities (Ohgushi, 2005; Stam *et al.*, 2014). In addition, communities belowground, including the microbiome around the roots, may also be an important component of plant defense (Berendsen *et al.*, 2012; Rasmann & Turlings, 2016). For instance, plants may recruit soil-borne entomopathogenic nematodes to their roots upon insect damage (Rasmann *et al.*, 2005) (Fig. **1, 2**). Moreover, soil microbes that assist in the defense against pathogens or insects may be recruited to the rhizosphere (Berendsen *et al.*, 2018; Hu *et al.*, 2018) (Fig. **1, 3**). Consequently, plants influence the community of beneficial organisms aboveground as well as belowground, thus enhancing their defense against various attackers.

Although plant-insect and plant-microbe interactions have mainly been studied separately, this is rapidly changing (Pieterse & Dicke, 2007; Rasmann *et al.*, 2017). Studies of microbe-plant-insect interactions show that each participant may have major effects on the interactions between the other organisms. Studies on the effects of microbes initially focused mainly on effects of individual bacterial species or strains on plant and insect performance (Hol *et al.*, 2013; Pangesti *et al.*, 2013) (Supplementary Table **S1**). The focus is currently changing to considering the full microbial community (Pineda *et al.*, 2017; Dini-Andreote & Raaijmakers, 2018; Cordovez *et al.*, 2019). Similarly, studies on the effects of insect herbivores have long focused on effects of individual

species, but now shift to a community approach as well (van Zandt & Agrawal, 2004; Utsumi *et al.*, 2013; Stam *et al.*, 2014; Poelman & Kessler, 2016; Ando *et al.*, 2017). Nevertheless, the bidirectional nature of microbe-plant-insect interactions has received little attention. Here, we will review current knowledge on the effects of beneficial rhizobacteria *via* alterations in plant defense traits on insects as well as the effects of plant defense against insects on the microbiome in the rhizosphere.

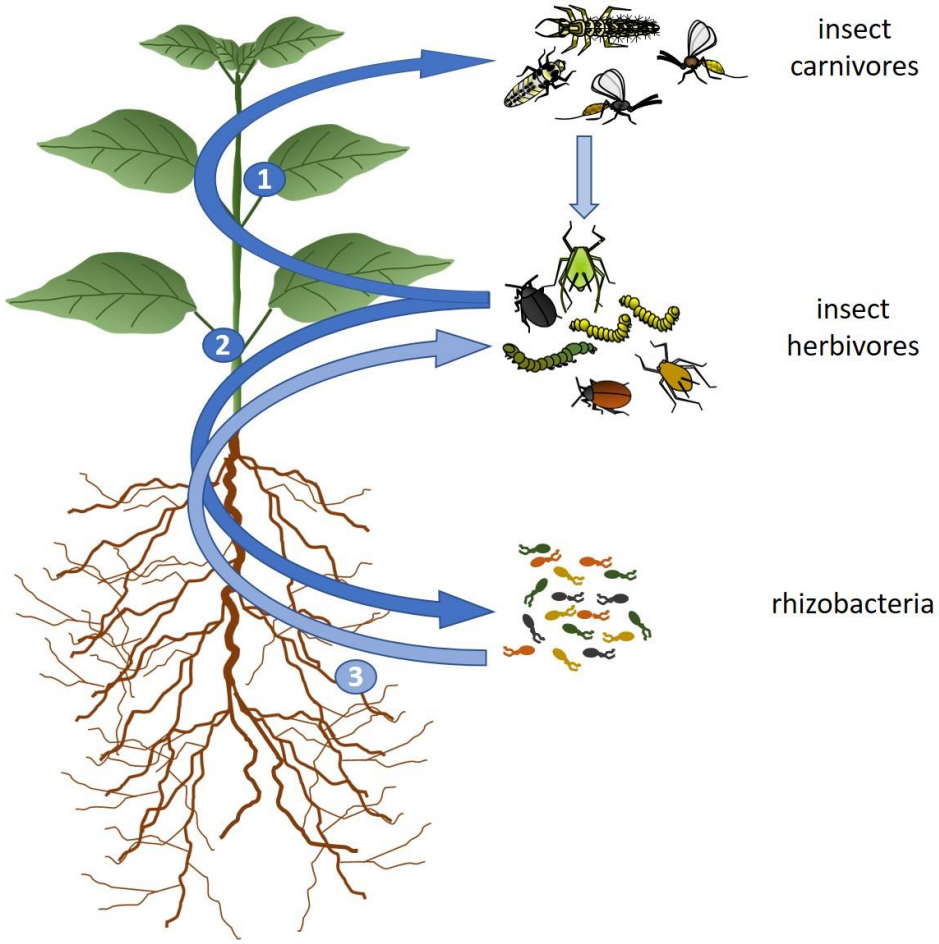

Figure 1 Plants may recruit other organisms that assist them in their defense. **1.** Upon insect herbivory on the shoot, plants produce herbivore-induced volatiles that attract parasitoids or predators that attack the herbivore (Stam *et al.*, 2014; Turlings & Erb, 2018); **2.** Upon insect feeding on the roots, plants may produce herbivore-induced volatiles that attract entomopathogenic nematodes (Rasmann *et al.*, 2005); **3.** Upon insect feeding plants may recruit rhizobacteria to the rhizosphere (Hu *et al.*, 2018).

Microbial community in the rhizosphere

The rhizosphere, the soil layer directly around the roots that is influenced by the roots, harbors a daunting number and variety of

microbes, up to tens of thousands of species. These microbes include pathogens and synergists that provide a wide diversity of services to plants (Mendes *et al.*, 2011; Berendsen *et al.*, 2012). Plants and their rhizosphere microbial community interact extensively.

Beneficial effects on plant growth and defense

Beneficial rhizobacteria, among which *Bacillus* spp. and *Pseudomonas* spp. have been most extensively studied, are characterized by their effects on plants such as growth promotion, increased nutrient uptake or enhanced plant defense (Pineda *et al.*, 2010; Pieterse *et al.*, 2014) and have therefore been termed plant growth-promoting rhizobacteria (PGPR). PGPR can promote plant growth through various mechanisms, including upregulation of photosynthesis, regulation of plant growth hormones or bacterial production of plant hormones or hormonal precursors (Pineda *et al.*, 2010). Physical contact between plant and bacteria is not always necessary, as rhizobacterial volatiles alone can increase chlorophyll and sugar content as well as photosynthetic efficiency (Ryu *et al.*, 2004; Zhang *et al.*, 2008). Beneficial rhizobacteria and fungi can also enhance plant resistance to a range of biotic stresses, increase nutrient uptake and inhibit access of pathogenic bacteria to the rhizosphere (Pieterse *et al.*, 2014). In real-time behavioral studies, colonization by *Bacillus subtilis* was observed to actively exclude *Escherichia coli* from the rhizosphere of *Arabidopsis thaliana* seedlings, probably *via* exudations from *B. subtilis* or the root (Massalha *et al.*, 2017). Moreover, beneficial rhizobacteria are known to generate changes in morphological features, such as root architecture, through stimulation of root hair formation and root elongation (Asari *et al.*, 2017).

Supplemental Table **S1** summarizes the effects of rhizobacteria on herbivorous insects, both directly and *via* plant-mediated effects as reported in the literature. Most studies have been conducted with bacterial strains of the genera *Bacillus* and *Pseudomonas*, mainly looking at effects on aphids and caterpillars. This highlights the need for studies evaluating rhizobacteria-plant-insect interactions in other systems that represent the wide microbial and insect diversity in nature. An interesting aspect is that the different applications such as soil drench, root or seed dip, can all result in effects on insects.

Root exudation influences rhizosphere community

Plants influence interactions with soil microorganisms through root-produced exudates that are disseminated into the surrounding soil (Berendsen *et al.*, 2012; Sharifi & Ryu, 2017). Root exudates can be released through both passive and active mechanisms (Badri & Vivanco, 2009; Huang, XF *et al.*, 2014). The amount of released exudates varies

along the root, with the highest levels around the root tip (Sasse *et al.*, 2018). Substantial information has become available on the many compounds exuded by roots, including primary metabolites, such as sugars and amino acids, as well as secondary metabolites, such as flavonoids, coumarins, glucosinolates, or plant hormones, and the response of the bacteria to them (Sasse *et al.*, 2018). Interestingly, many of the compounds found in root exudates are common soil compounds (Dennis *et al.*, 2010) and most of the root-exudate components are metabolized by microbes (Berendsen *et al.*, 2012). Root exudates are an important source of carbon for soil microbes and exudate composition differs between plant species and even plant cultivars, indicating that plants support distinct groups of microbes (Smalla *et al.*, 2001; Bulgarelli *et al.*, 2013).

Bacterial chemotaxis to root exudates

Bacteria are attracted to the rhizoplane through chemotaxis. For instance, exudates from *A*. *thaliana* roots attract *B*. *subtilis* (Allard-Massicotte *et al.*, 2016) to the border region between the root elongation and early-maturation zones (Massalha *et al.*, 2017). Rice root exudates attract rhizobacteria (*Azospirillum brasilense* and *Bacillus sp*.) (Bacilio-Jimenez *et al.*, 2003). Furthermore, benzoxazinoids in maize-root exudates attract *Pseudomonas putida* (Neal et al., 2012). Root extracts of banana (Yuan *et al.*, 2015b), tobacco (Wu *et al.*, 2015) and cucumber (Zhang *et al.*, 2014) have also been reported to attract bacteria. Flagella-mediated motility is an important characteristic of bacteria to reach the root (Allard-Massicotte *et al.*, 2016). However, colonization seems to be possible even without such motility, as experiments with motility-impaired bacterial mutants still demonstrate colonization capacity (Maldonado-Gonzalez *et al.*, 2015).

Biofilm formation and quorum sensing

Root colonization by rhizobacteria quickly results in the formation of a biofilm around the root. After only six hours of contact, *B*. *subtilis* formed a biofilm on *A*. *thaliana* roots (Allard-Massicotte *et al.*, 2016). This biofilm protects the roots against pathogens (Kim & Anderson, 2018). The event is mediated by extensive plant-bacteria communication, including the involvement of plant root exudates (Drogue *et al.*, 2012). Biofilm formation by *B*. *subtilis* on *A*. *thaliana* roots was promoted by the release of plant polysaccharides (Beauregard *et al.*, 2013). Microbe-microbe interactions important for biofilm development are regulated by cell-to-cell communication signals, diffusible N-acyl-homoserine lactones, which function as quorum-sensing signals. These signals activate hundreds of

bacterial genes, including genes involved in biofilm formation, nitrogen fixation, synthesis of degrading enzymes, exopolysaccharides, and toxins, as well as motility and conjugation (Hassan & Mathesius, 2012).

Some plants have the ability to produce compounds mimicking quorum-sensing signals, thus promoting quorum sensing and stimulating biofilm formation. In contrast, other plants inhibit quorum-sensing signals (Kan *et al.*, 2017). Furthermore, bulk soil bacteria and rhizobacteria exhibit different responses to plant exudates. Two strains of the growth-promoting root colonizer *Bacillus mycoides*, one a root isolate and the other a soil isolate, contrasted clearly in gene regulation in response to potato root exudates (Yi *et al.*, 2017). The density of rhizobacteria needed to induce plant defense was found to be 10^5 colony forming units (CFU) per gram root tissue for *Pseudomonas spp*. on radish *Raphanus sativus* roots (Raaijmakers *et al.*, 1995). However, the surface area on the root needed for plant defense activation, and how the rhizobacteria interact with other root colonizers such as mycorrhiza or endophytic bacteria is largely unknown (Carrion *et al.*, 2019).

Insect community associated with plants

At present about one million insect species have been identified, of which approximately half consist of herbivores (Schoonhoven *et al.*, 2005). An individual plant may face the attack of hundreds of insect species, each of which is attacked by a diversity of carnivorous insects. Thus, plants are members of a diverse insect community consisting of species at different trophic levels (Stam *et al.*, 2014). Plants interact with this community both directly and indirectly (Morris *et al.*, 2007; Rasmann *et al.*, 2011; Stam *et al.*, 2014; Turlings & Erb, 2018).

Induced plant defense against insects

Plants have diverse defense strategies against herbivorous insects. These strategies can be categorized into direct and indirect defenses. Direct defense consists of traits that negatively affect herbivores, whereas indirect defense involves the promotion of the effectiveness of natural enemies of herbivores, e.g. through their attraction or arrestment (Stam *et al.*, 2014). For instance, upon herbivory brassicaceous plants activate glucosinolate production (direct defense) (Gols *et al.*, 2008) as well as the emission of a blend of volatiles that attract natural enemies of the herbivores (Mattiacci *et al.*, 1995). Roots may also emit herbivore-induced volatiles that attract soil-borne natural enemies of root-feeding insects such as entomopathogenic nematodes (Rasmann *et al.*, 2005; van Dam & Bouwmeester, 2016).

Such induced plant defenses against herbivorous insects involve both local and systemic plant traits. Plant hormones regulate induced defenses (Pieterse *et al.*, 2012; Stam *et al.*, 2014). Two major pathways involved in regulating induced defenses are the salicylic acid (SA) pathway and the combined jasmonic acid (JA) - ethylene (ET) pathway (Pieterse *et al.*, 2012; Verma *et al.*, 2016). Additionally, other hormones such as abscisic acid (ABA), auxin, cytokines (CK) and gibberellic acid (GA) may be involved in the regulation of plant responses to biotic stressors (Pieterse *et al.*, 2012). The feeding mode of the insect herbivore affects the signaling pathway that is activated: the JA pathway is generally induced in response to biting-chewing insects, such as caterpillars or beetle larvae, whereas the SA pathway is especially induced by piercing-sucking phloem feeders. The phytohormonal signaling pathways do not function independently from each other. Cross-talk between them allows plants to fine-tune defense responses (Pieterse *et al.*, 2012; Verma *et al.*, 2016). As a result of phytohormone induction, insect herbivory alters a plant's phenotype species-specifically both locally and systemically, in terms of physical as well as chemical aspects.

Insect-induced changes in plant phenotype affect rhizobacterial community

Insect-induced changes in root exudates

One of the phenotypic changes induced by insect herbivory is the composition of plant root exudates. Maize plants increase their carbon allocation to roots and root exudates upon feeding by the lubber grasshopper *Romalea guttata* (Holland *et al.*, 1996) and *Spodoptera littoralis* infestation of maize seedlings increases the release of benzoxazinoids by the roots (Marti *et al.*, 2013; Hu *et al.*, 2018). Phytohormonal induction in response to herbivory may underlie root exudate composition. Root exudation may include the release of phytohormones into the rhizosphere as has been found for many plant species (Li *et al.*, 2016b; Kong *et al.*, 2018). After experimental treatment with plant hormones some rhizobacterial communities changed (Carvalhais *et al.*, 2014), whereas others remained unchanged (Santhanam *et al.*, 2014; Liu *et al.*, 2017).

The importance of phytohormonal signaling pathways for root exudation has been further demonstrated in mutants: *A. thaliana* mutants defective in particular defense signaling pathways had a diverging rhizobacterial community (Kniskern *et al.*, 2007; Hein *et al.*, 2008; Doornbos *et al.*, 2009; Lebeis *et al.*, 2015). For instance, the *A. thaliana* mutants *myc2* and *med25* which are impaired in the JA-signaling pathway

had different exudate compositions compared to wild type plants (Carvalhais *et al.*, 2015).

Moreover, a plant may adjust its exudate depending on the rhizobacteria present in the rhizosphere, both quantitatively and qualitatively (Huang, XF *et al.*, 2014). The protein composition in the exudate secreted from the roots of *Medicago sativa* and *A. thaliana* changed depending on the identity of two rhizosphere bacteria, a symbiont or a pathogen (De-La-Pena *et al.*, 2008). In conclusion, the composition of root exudates is influenced by herbivorous insects that attack the plant. Because root exudates influence microbiome composition, this suggests that herbivory affects the rhizosphere microbial community.

Herbivore-induced changes in root-associated microbiome

Indeed, herbivore infestation of plants results in changes in the root-associated microbiome. Barrelclover, *Medicago truncatula*, forms more associations with rhizobia after infestation with *S. exigua* caterpillars. In response to attack by the whitefly *Bemisia tabaci*, pepper plants were colonized most abundantly by *Achromobacter*, *Janthinobacterium* and *Stenotrophomonas* rhizobacteria (Kong *et al.*, 2016). The latter bacterial genus was also found in a study on the response of *A. thaliana* to infection by the pathogen *Hyaloperonospora arabidopsidis* (Berendsen *et al.*, 2018). Feeding by *S. littoralis* caterpillars on maize seedlings influenced the rhizosphere microbiome (Hu *et al.*, 2018). Interestingly, aboveground and belowground herbivory of ragwort plants *Jacobaea vulgaris* differentially influenced soil microbiota, especially soil fungi (Kostenko *et al.*, 2012). Herbivores may even affect root-microbe interactions before the herbivores initiate continuous feeding. In an interesting experiment, the exposure of alder *Alnus spp*. plants to woolly alder aphids *Prociphilus tessallatus* that did not initiate feeding, resulted in increased nitrogen fixing activity within a day (Zekveld & Markham, 2011). These effects of herbivory on root-associated microbes may be mediated by root exudates. Benzoxazinoids are known to attract *Pseudomonas putida* to maize roots (Neal *et al.*, 2012). Enhanced exudation of benzoxazinoids by maize plants in response to caterpillar feeding on the leaves influenced the rhizosphere composition of bacteria and fungi; this effect was mediated by the benzoxazinoid breakdown product 6-methoxy-benzoxazolin-2-one (MBOA) as was shown by comparing wildtype plants and benzoxazinoid mutants in combination with complementation of MBOA (Hu *et al.*, 2018).

Even when the total number of rhizobacteria remains the same, different bacterial groups may be differentially affected by insect-

herbivory mediated changes in plant phenotype, thus resulting in a change in community composition (Hu *et al.*, 2018). For instance, the population density of gram-positive bacteria, but not the total density of bacteria, on pepper roots *Capsicum annuum* increased upon feeding by the whitefly *B. tabaci* (Yang *et al.*, 2011). Similarly, gram-positive rhizobacteria increased after feeding by the green peach aphid *Myzus persicae* on the shoots of pepper plants (Kim *et al.*, 2016). Single bacterial strains have been observed to be specifically supported by the plant after insect attack. The density of the growth-promoting *B. subtilis* increased on pepper roots upon infestation by *M. persicae* (Lee *et al.*, 2012). Furthermore, pepper roots restructured rhizosphere microbiota composition after one week of *B. tabaci* infestation, favoring Pseudomonadales, such as the genus *Pseudomonas* (Kong *et al.*, 2016). The impact of root herbivores is less well studied, but infestation of maize roots with Western corn rootworm larvae, *Diabrotica virgifera virgifera*, resulted in an increase of *Acinetobacter calcoaceticus* in the rhizosphere (Dematheis *et al.*, 2012). In conclusion, insect herbivory may alter the community of the rhizosphere microbiome through changes in root exudate composition. Because many rhizosphere microbes are known to affect plant resistance to insect herbivores, the effects of the altered rhizosphere microbial community on plant defense are an interesting next step in understanding microbe-plant-insect interactions.

Effects of herbivory-induced changes in rhizobacteria community on insect herbivores

Plant-mediated effects of rhizobacteria on insect herbivores

Rhizobacteria are known to modulate induced plant defense through a mechanism called priming. Priming is a phenomenon that does not result in defense gene upregulation or biosynthesis of defensive secondary metabolites, but rather triggers a state that allows for a faster and/or more intense defense response once the attack occurs (Conrath *et al.*, 2006; Hilker *et al.*, 2016; Martinez-Medina *et al.*, 2016). For example, after priming, systemic signaling molecules may be pre-synthesized as conjugates ready to be activated by herbivory (Pastor *et al.*, 2013). The fitness costs of priming can be lower than activation of defense upon herbivore attack (van Hulten *et al.*, 2006), the costs of which can be considerable (Balmer *et al.*, 2015). Rhizobacterial priming of induced systemic resistance (ISR) is commonly dependent on intact JA signaling (Pieterse *et al.*, 2014). However, *Pseudomonas fluorescens* strain SS101 can mediate ISR *via* the SA pathway in *A. thaliana* (van de Mortel *et al.*, 2012). Rhizobacterially mediated priming of plant defense has been especially reported for defense against plant pathogens

(Pieterse *et al.*, 2014; Mauch-Mani *et al.*, 2017), but can also be effective against insect herbivores (Kim & Felton, 2013; Pangesti *et al.*, 2015a; Pangesti *et al.*, 2015b; Hilker *et al.*, 2016). Studies on rhizobacterial priming of defense against insect herbivores are rapidly gaining interest.

Rhizobacterial colonization of plant roots may affect oviposition preference of insect herbivores. For instance, the European corn borer moth *Ostrinia nubilalis* prefers to oviposit on control plants compared to plants with rhizobacterial colonization (Disi *et al.*, 2018). Rhizobacteria can also influence feeding herbivores (Pineda *et al.*, 2010). The effect of rhizobacterial colonization of plant roots on an insect herbivore feeding on a plant can be both positive and negative. For instance, aphid performance can be enhanced on plant with rhizobacterial colonization of the roots (Dean *et al.*, 2009; Pineda *et al.*, 2012; Megali *et al.*, 2014; Naluyange *et al.*, 2014; Kim *et al.*, 2016), but also negative (Gadhave *et al.*, 2016a) or neutral effects (Herman *et al.*, 2008; Dean *et al.*, 2014) have been recorded. Rhizobacterial colonization may suppress whitefly populations on tomato plants (Murphy *et al.*, 2000; Valenzuela-Soto *et al.*, 2010) and pepper plants (Chale-Carrillo *et al.*, 2016). In contrast, whitefly populations increase after rhizobacterial inoculation of tomato (Shavit *et al.*, 2013). The effects of rhizobacteria on insect herbivores may be dependent on soil nutrient conditions (Pangesti *et al.*, 2015a). These data indicate that variation in plant responses occurs.

An interesting study on insect-maize-microbe interactions shows that changes in rhizosphere microbial communities as a result of feeding by *S*. *littoralis* caterpillars on maize plants enhance plant resistance to *S*. *littoralis*. This effect is mediated by *S*. *littoralis*-induced MBOA secretion by the maize roots and JA signaling in the plant (Hu *et al.*, 2018). This study is the first to indicate that herbivore-induced changes in the rhizosphere microbial community feed back to plant resistance against an insect herbivore. This raises the question whether such feed-back effects influence the community of insects associated with plants.

Effects of rhizobacteria on the plant-associated insect community

In the context of effects of rhizobacteria on plant resistance to individual herbivorous insect species, it is interesting to note that a single rhizobacterium can differentially influence various insect herbivores. For instance, colonization of *A*. *thaliana* roots by *Pseudomonas simiae* WCS417r (formerly *P*. *fluorescens* WCS417r) results in negative effects on the performance of the generalist caterpillars *S. exigua* and *Mamestra brassicae*, positive effects on the generalist aphid *M*. *persicae*, and neutral effects on the specialist aphid *Brevicoryne brassicae* and the specialist caterpillar *Pieris rapae* (van Oosten *et al.*, 2008; Pineda *et al.*, 2012;

Pangesti *et al.*, 2015a) (Fig. **2**). This suggests that rhizobacteria influence the insect herbivore community associated with plants. Rhizobacteria can also influence tritrophic species interactions *via* herbivore-induced plant volatiles (Pineda *et al.*, 2010; Rasmann *et al.*, 2017). These effects also appear to be species specific. For instance, adding *Bacillus* spp. to the soil increases the parasitism rates of the cabbage aphid *B*. *brassicae* by the parasitic wasp *Diaeretiella rapae* on *Brassica oleracea* plants, but did not increase ladybird beetle *Coccinella septempunctata* and syrphid fly feeding on the aphids (Gadhave *et al.*, 2016a). In contrast, natural parasitism levels of cabbage aphid *B*. *brassicae* correlate negatively with the abundance of *Bacillus ssp*. when broccoli plants are grown in soils with naturally different amounts of bacteria (Blubaugh *et al.*, 2018). *Pseudomonas simiae* WCS417r colonization of *A*. *thaliana* roots results in reduced attraction of the aphid parasitoid *Diaeretiella rapae* in response to infestation with the aphid *M*. *persicae*, whereas it enhanced attraction of the parasitoid *Microplitis mediator* upon feeding damage by *M*. *brassicae* caterpillars (Pineda *et al.*, 2013; Pangesti *et al.*, 2015b) (Fig. **2**). Thus, colonization by a single rhizobacterium differentially affects plant-associated insects at not only the second level but also at the third trophic level. Part of these effects are caused by modification of herbivore-induced plant volatile emission as a result of plant-root-microbe interactions, with consequences for insect behavior (Ballhorn *et al.*, 2013). In a field experiment, rice plants treated with *Pseudomonas fluorescens* exhibited an increase in natural enemies of herbivores, especially hymenopteran parasitoids and spiders (Commare *et al.*, 2002). It remains to be investigated what caused this effect.

Altogether, these findings suggest that rhizobacterial colonization of plant roots may influence the insect community. Investigating this with a focus on the underlying mechanisms to understand how rhizobacteria influence insect community processes and the consequences for plant performance will be important to enhance our understanding of the ecological effects of herbivory-induced changes on rhizobacteria mediated effects on the plant-associated insect community.

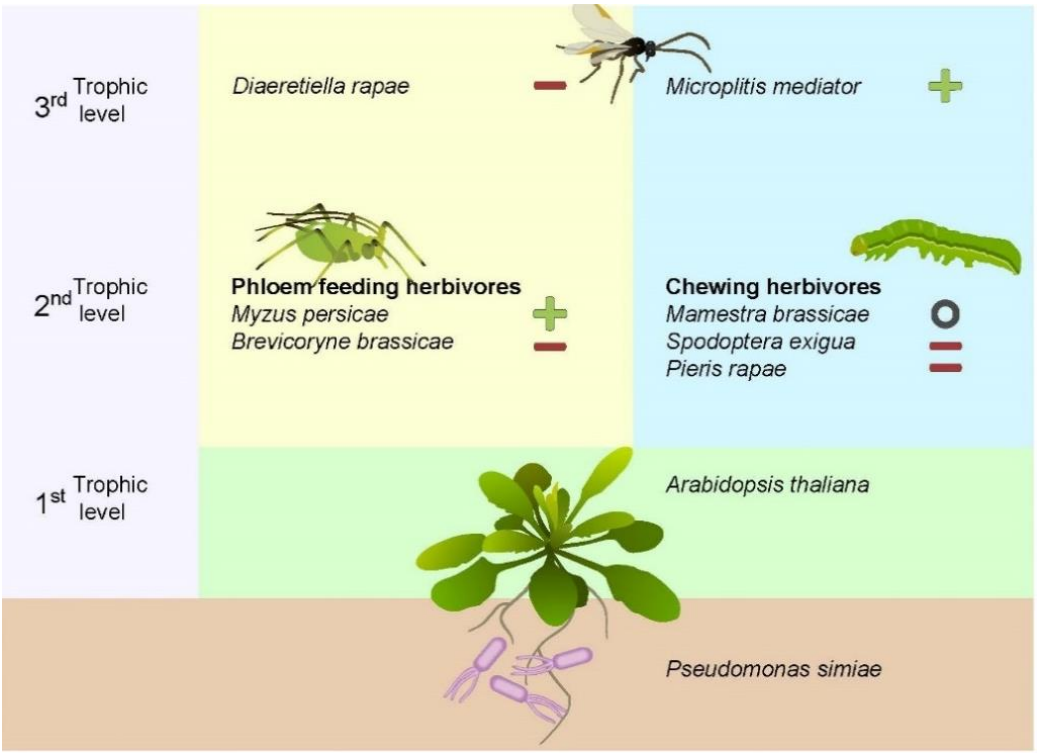

Figure 2 Differential effects of the Plant-Growth Promoting Rhizobacterium *Pseudomonas simiae* WCS417 on insect herbivores and their parasitoids on the host plant *Arabidopsis thaliana*. Green + indicates beneficial effect on insect performance, red – indicates detrimental effects on insect performance and grey o indicates neutral effects on insect performance. Figure based on van Oosten *et al.* (2008); Pineda *et al.* (2012); Pineda *et al.* (2013); Pangesti *et al.* (2015a); Pangesti *et al.* (2015b).

Concluding remarks and future perspectives

Plants are known to recruit the enemies of insect herbivores by emitting herbivore-induced plant volatiles in response to attack (Dicke & Baldwin, 2010). The present review has presented the emerging view that plants may also recruit rhizosphere microbes that enhance the plant's defense against insect herbivores. Maize plants respond to feeding by *S. littoralis* caterpillars with the emission of herbivore-induced root exudate that alters the rhizosphere microbiome, thus enhancing plant resistance to the caterpillars in previously undamaged plants (Hu et al., 2018). This plant-soil feedback is even more complex than indirect plant defense mediated by herbivore-induced plant volatiles, because it involves a plant response to the recruited microbes. The ecology of PGPR is a recent field of research that advances at high pace (Balmer *et al.*, 2015; Sharifi & Ryu, 2017; Hu *et al.*, 2018; Carrion *et al.*, 2019). With new data showing that plants may recruit rhizobacteria that contribute to their defense

(Kostenko *et al.*, 2012; Pineda *et al.*, 2017; Berendsen *et al.*, 2018; Hu *et al.*, 2018), just like they recruit beneficial entomopathogenic nematodes belowground or predators and parasitoids aboveground (Dicke & Baldwin, 2010; Turlings & Erb, 2018), plant defense against insects appears to be a participatory activity involving selected members of the associated community.

Initial studies have addressed the plant-mediated effects of rhizobacteria on insect herbivores and carnivores (Pineda *et al.*, 2010; Pineda *et al.*, 2013; Pangesti *et al.*, 2015b; Rasmann *et al.*, 2017; Hu *et al.*, 2018). The first studies show that rhizobacteria differentially affect members of the plant-associated insect community. So far, that information is restricted to a limited number of herbivorous and carnivorous insects. No information is available on such effects of rhizobacteria on other community members such as pollinators. Gaining information on this is relevant because pollinators directly contribute to reproductive success of many plant species (Rusman *et al.*, 2019). Phenotypic plasticity in plants as a result of interactions with community members such as insect herbivores may influence pollinator activity and it is likely that phenotypic plasticity resulting from interactions with herbivory-induced changes in rhizosphere microbes does so as well. Indeed, other soil microbiota, such as arbuscular mycorrhizal fungi are known to affect aboveground flower traits such as number of flowers per plant, inflorescence size or nectar content with consequences for pollinator behavior (Gange & Smith, 2005). Investigating whether herbivory-induced changes in PGPR also influence plant-pollinator interactions will be important to understand the consequences of plant-PGPR interactions in the context of herbivory-induced rhizobacteria recruitment in terms of plant fitness consequences.

In community ecology, dispersal has been implied as one of the main drivers of community transformation (Dini-Andreote & Raaijmakers, 2018). PGPR may disperse on plant seeds (Truyens *et al.*, 2015; Berg & Raaijmakers, 2018). There might be a relationship between the seed microbiome composition and plant pathogen defense (Rybakova *et al.*, 2017), but it is not yet clear if this extends to defense against insects. Also, it is not clear how plant attack by insects affects the incorporation of microbes into the seed of the parent plant, or how herbivory-induced phenotypic changes impact microbial transfer to the seed. Moreover, PGPR have been recorded in the gut of the diamondback moth *Plutella xylostella*, a specialist insect herbivore, suggesting that insects may also play a role in bacterial dispersal (Indiragandhi *et al.*, 2008).

Many questions regarding the temporal and spatial dynamics of herbivore-induced bacterial communities, especially of plants exposed to

abiotic and biotic stresses still remain unresolved (Berendsen *et al.*, 2012; Aznar & Dellagi, 2015). Plant developmental stage has been shown to influence both the insect community and the plant associated microbiome, but the two factors have not yet been linked.

Overall, new information indicates that plants actively recruit and retain specific bacterial groups to enhance plant defense. Therefore, the increase of specific rhizobacteria associated with insect attack might be regarded as part of the plant's inducible defense. In this review it has been shown that the rhizosphere is influenced by individual herbivore species feeding on a plant. In nature, plants are commonly attacked by a diversity of insect species (Stam *et al.*, 2014). The effects of multiple attackers on a single plant interact, e.g. *via* crosstalk of plant signal-transduction pathways. This may lead to synergistic and antagonistic effects on the plant's phenotype, with consequences for the members of the associated insect community (Stam *et al.*, 2014). How multiple attack influences the plant's effects on the rhizosphere microbiome remains to be investigated.

As plants are central members of a diverse community including insects and microbes aboveground and belowground, plant attackers and their antagonists, plant-mediated interactions are important forces shaping plant-associated community ecology and influencing plant performance (Stam *et al.*, 2014). Incorporating bidirectional effects of beneficial rhizobacteria and herbivorous insects on plant-mediated interactions within the plant-associated community will increase our understanding of plant ecology in a community context. Such knowledge may be used to develop agricultural practices that exploit ecological interactions to produce crops without input of chemical pesticides.

Author contributions

JF executed the literature analysis, prepared the table, and wrote the first draft of the paper. JF and MD prepared the figures. AP, JJAvL and MD significantly contributed to the finished manuscript. All authors read and approved the manuscript.

Supplemental information

Table S1 (next pages) Overview of the effects of individual rhizobacteria on insect herbivores, both directly and indirectly *via* plant-mediated effects. Green boxes represent legume species. Bacterial mixes are displayed as a list in one cell in the column Bacteria. N/a: Not available. A - indicates a negative effect on the insect through the addition of bacteria, whereas + indicates a positive effect on the insect.

Insect	Plant	Growth conditions	Root substrate	Bacteria	Bacterial application	Growth	Developmental time (- indicates longer time)
Beet armyworm *Spodoptera exigua*	*Arabidopsis thaliana*	Growth chamber	Agar	*Pseudomonas fluorescens* SS101	Root tip inoculation		
Beet armyworm *Spodoptera exigua*	Cotton *Gossypium hirsutum*	Growth chamber	Soil	*Bacillus pumilis* INR-7	Spore powder formulation + soil drench	-	
Beet armyworm *Spodoptera exigua*	Cotton *Gossypium hirsutum*	Growth chamber	Soil	*Bacillus amyloliquefaciens* AP-188 *Bacillus mojavensis* AP-217 *Bacillus solisalsi* AP-209 *Bacillus amyloliquefaciens* AP-218	Spore powder formulation + soil drench	-	
Beet armyworm *Spodoptera exigua*	Cotton *Gossypium hirsutum*	Growth chamber	Soil	*Bacillus amyloliquefaciens* AP-136 *Bacillus amyloliquefaciens* AP-188 *Bacillus amyloliquefaciens* AP-219 *Bacillus amyloliquefaciens* AP-295	Spore powder formulation + soil drench	-	
Cabbage aphid *Brevicoryne brassicae*	*Arabidopsis thaliana*	Growth chamber	Soil	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	+	
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Field	Field soil	*Bacillus cereus* 8FW	Applied on seeds		
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Field	Field soil	*Bacillus subtilis* NRRLB23051	Applied on seeds		
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Field	Field soil	*Bacillus amyloliquefaciens* FZB42BGSC10A6	Applied on seeds		
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Growth chamber	Soil	*Bacillus cereus* 8FW	Soil drench		-
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Growth chamber	Soil	*Bacillus subtilis* NRRLB23051	Soil drench		No effect
Cabbage aphid *Brevicoryne brassicae*	Calabrese *Brassica oleracea*	Growth chamber	Soil	*Bacillus amyloliquefaciens* FZB42BGSC10A6	Soil drench		-
Cabbage looper *Trichoplusia ni*	*Arabidopsis thaliana*	Growth chamber	Field soil	Natural microbiome	Soil drench	N/a	N/a
Cabbage moth *Mamestra brassicae*	*Arabidopsis thaliana*	Growth chamber	Soil/sand	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	+	
Cabbage moth *Mamestra brassicae*	*Arabidopsis thaliana*	Growth chamber	Soil/sand	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	-/+	
Cabbage moth *Mamestra brassicae*	*Arabidopsis thaliana*	Growth chamber	Soil/sand	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	+	
Cabbage moth *Mamestra brassicae*	*Arabidopsis thaliana*	Growth chamber	Agar	*Pseudomonas (fluorescens) simiae* WCS417	Root tip inoculation	-	
Cabbage root fly *Delia radicum*	Rapeseed *Brassica napus*	Greenhouse	Field soil	Natural microbiome	High and low concentration of microbiome	N/a	N/a
Corn rootworm *Diabrotica speciosa*	Maize *Zea mays*	Greenhouse	Soil	*Azospirillum brasilense* AbV5 *Azospirillum brasilense* AbV6	Applied on seeds	-	
Cotton aphid *Aphis gossypii*	Cucumber *Cucumis sativus*	Greenhouse	Soil	*Pseudomonas fluorescens* UTPF68	Applied on seeds		No effect
Cotton aphid *Aphis gossypii*	Cucumber *Cucumis sativus*	Greenhouse	Soil	*Pseudomonas fluorescens* UTPF1	Applied on seeds		+
Cotton aphid *Aphis gossypii*	Cucumber *Cucumis sativus*	Greenhouse	Soil	*Pseudomonas fluorescens* UTPF6	Applied on seeds		No effect
Cotton aphid *Aphis gossypii*	Cucumber *Cucumis sativus*	Greenhouse	Soil	*Pseudomonas fluorescens* PF169	Applied on seeds		No effect
Cotton aphid *Aphis gossypii*	Okra *Abelmoschus esculentus*	Field	Sandy loam	*Pseudomonas fluorescens* PF1	Applied on seeds		
Cotton aphid *Aphis gossypii*	Squash *Cucurbita pepo*	Growth chamber	Soil/sand	*Rhizobium etli* G12	Soil drench		
Cotton aphid *Aphis gossypii*	Tomato *Solanum lycopersicum*	Field	Soil	*Bacillus subtilis* BS3A25	Applied on seeds		-
Cotton aphid *Aphis gossypii*	Tomato *Solanum lycopersicum*	Field	N/a	*Bacillus subtilis* BS3A25	Foliar spray		
Cotton leafworm *Spodoptera littoralis*	Maize *Zea mays*	Growth chamber	Soil	*Enterobacter aerogenes*	Root dip	+	
Cotton leafworm *Spodoptera littoralis*	Tomato *Solanum lycopersicum*	Greenhouse	Soil	*Lactobacillus plantarum* *Lactobacillus casei* *Streptococcus lactis* *Saccharomyces* spp. *Rhodopseudomonas plastris* *Rhodobacter sphacrodes* *Streptomyces* spp.	Soil drench	+	
Cotton leafworm *Spodoptera littoralis*	Corn *Zea mays*	Greenhouse	Soil	*Lactobacillus plantarum* *Lactobacillus casei* *Streptococcus lactis* *Saccharomyces* spp. *Rhodopseudomonas plastris* *Rhodobacter sphacrodes* *Streptomyces* spp.	Soil drench	+	

Pupation rate	Number/ plant	Survival	Fecundity	Intrinstic rate of increase (r_m)	Rate of increase	Oviposition preference	Consumed plant tissue (- indicates higher plant resistance)	Bacterial number assessed at the end of experiment?	Insect effect on bacteria	Reference	Remarks
		-						Yes	N/a	van de Mortel et al., 2012	
-		-						No	N/a	Zebelo et al., 2016	
-		-						No	N/a	Zebelo et al., 2016	
-		-						No	N/a	Zebelo et al., 2016	
				+				Yes	N/a	Pineda et al. 2012	
	-							Yes	N/a	Gadhave et al., 2016a	
	-							Yes	N/a	Gadhave et al., 2016a	
	-							Yes	N/a	Gadhave et al., 2016a	
			-	-	-			No	N/a	Gadhave et al., 2016b	
			No effect	No effect	-			No	N/a	Gadhave et al., 2016b	
			No effect	-	-			No	N/a	Gadhave et al., 2016b	
N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	Yes	Abundance of some bacterial families was linked to lower insect feeding	Badri et al., 2013	
								No	N/a	Fernandez de Bobadilla et al., 2017	
								Yes	N/a	Pangesti et al., 2015a	Nutrient-rich soil = positive/negative depending on batch of soil Nutrient-poor soil = negative
								Yes	N/a	Pangesti et al., 2015b	
								Yes	N/a	Pangesti et al., 2016	
N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	Yes	Bacterial community altered, increase of Bacillus, Clostridium, Paenibacillus, and Pseudomonas	Ourry et al., 2018	
								Confirmed in other experimental plants	N/a	Santos et al., 2014	
				-	-			No	N/a	Fahimi et al., 2014	
				+	No effect			No	N/a	Fahimi et al., 2014	
				+	-			No	N/a	Fahimi et al., 2014	
				-	-			No	N/a	Fahimi et al., 2014	
	-							No	N/a	Gandhi et al., 2006	
	-							No	N/a	Martinuz et al., 2012	
				-	-			No	N/a	Sudhakar et al., 2011	
	-							No	N/a	Sudhakar et al., 2011	
							No effect	Confirmed in other experimental plants	N/a	D'Alessandro et al., 2014	
		+						No	N/a	Megali et al., 2014	
								No	N/a	Megali et al., 2015	

Diamondback moth *Plutella xylostella*	Kale *Brassica oleracea*	Greenhouse	N/a	*Kluyvera ascorbata* EN4	Foliar spray	-	-
English grain aphid *Sitobion avenae*	Barley *Hordeum vulgare*	Greenhouse	Sand	*Pseudomonas aeruginosa* 7NSK3	Root dip		
English grain aphid *Sitobion avenae*	Barley *Hordeum vulgare*	Greenhouse	Sand	*Pseudomonas aeruginosa* 7NSK2	Root dip		
European corn borer *Ostrinia nubilalis*	Maize *Zea mays*	Growth chamber	Soil	*Bacillus pumilus* INR-7	Applied on seeds	No effect	
European corn borer *Ostrinia nubilalis*	Maize *Zea mays*	Growth chamber	Soil	*Bacillus velezensis* AP-188 *Bacillus mojavensis* AP-209 *Fictibacillus solisalsi* AP-217 *Bacillus velezensis* AP-218	Applied on seeds	No effect	
European corn borer *Ostrinia nubilalis*	Maize *Zea mays*	Growth chamber	Soil	*Bacillus velezensis* AP-136 *Bacillus velezensis* AP-188 *Bacillus velezensis* AP-219 *Bacillus velezensis* AP-295	Applied on seeds	No effect	
Green peach aphid *Myzus persicae*	*Arabidopsis thaliana*	Growth chamber	Soil/sand	*Pseudomonas (fluorescens) simiae* WCS417	Soil drench		
Green peach aphid *Myzus persicae*	*Arabidopsis thaliana*	Growth chamber	Field soil	Natural microbiome *Pseudomonas fluorescens* SS101	Mixed into soil		No effect
Green peach aphid *Myzus persicae*	*Arabidopsis thaliana*	Growth chamber	Soil	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	No effect	
Green peach aphid *Myzus persicae*	Pepper *Capsicum annuum*	Greenhouse	Field soil	Natural microbiome	N/a	N/a	N/a
Green peach aphid *Myzus persicae*	Pepper *Capsicum annuum*	Field	Field soil	*Bacillus subtilis* B03 *Bacillus amyloliquefaciens* IN937a	Mixed into soil		
Green peach aphid *Myzus persicae*	Pepper *Capsicum annuum*	Growth chamber	N/a	*Paenibacillus polymyxa* E681	Root dip		
Indian cotton jassid *Amrasca biguttula*	Okra *Abelmoschus esculentus*	Field	Sandy loam	*Pseudomonas fluorescens* PF1	Applied on seeds		
Large cabbage butterfly *Pieris brassicae*	*Arabidopsis thaliana*	Growth chamber	Soil/sand	*Pseudomonas (fluorescens) simiae* WCS417	Mixed into soil	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Greenhouse	Clay soil	*Pseudomonas fluorescens* PF1	Applied on seeds	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Greenhouse	Clay soil	*Pseudomonas fluorescens* FP7	Applied on seeds	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Field	Field soil	*Pseudomonas fluorescens* PF1	Applied on seeds		
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Field	Field soil	*Pseudomonas fluorescens* FP7	Applied on seeds		
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Greenhouse	Soil	*Pseudomonas fluorescens* PF1	Rehydrated bacteria in root dip	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Field	Soil	*Pseudomonas fluorescens* PF1	Rehydrated bacteria in root dip		
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Greenhouse	Soil	*Pseudomonas fluorescens* TDK1	Rehydrated bacteria in root dip	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Field	Soil	*Pseudomonas fluorescens* TDK1	Rehydrated bacteria in root dip		
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Greenhouse	Soil	*Pseudomonas fluorescens* PY15	Rehydrated bacteria in root dip	-	
Leaffolder *Cnaphalocrocis medinalis*	Rice *Oryza sativa*	Field	Soil	*Pseudomonas fluorescens* PY15	Rehydrated bacteria in root dip		
Silverleaf whitefly *Bemisia argentifolii*	Tomato *Solanum lycopersicum*	Field	Sand	*Bacillus amyloliquefaciens*	Applied on seeds		
Silverleaf whitefly *Bemisia argentifolii*	Tomato *Solanum lycopersicum*	Field	Sand	*Bacillus amyloliquefaciens*	Spore powder formulation added to the planting medium		
Silverleaf whitefly *Bemisia argentifolii*	Tomato *Solanum lycopersicum*	Field	Sand	*Bacillus subtilis*	Applied on seeds		
Silverleaf whitefly *Bemisia argentifolii*	Tomato *Solanum lycopersicum*	Field	Sand	*Bacillus subtilis*	Spore powder formulation added to the planting medium		
Spotted cucumber beetle *Diabrotica undecimpunctata*	Cucumber *Cucumis sativus*	Greenhouse	N/a	*Bacillus pumilus* INR-7	Applied on seeds		
Striped cucumber beetle *Acalymma vittata*	Cucumber *Cucumis sativus*	Field	Field soil	*Serratia marcescens*	Applied on seeds + soil drench		
Tobacco cutworm *Spodoptera litura*	Cabbage *Brassica oleracea*	Greenhouse	Field soil	*Burkholderia phytofirman* *Rhizobium miluonens* *Rhizobium lusitanum*	Root dip + soil drench		
Tobacco cutworm *Spodoptera litura*	Tomato *Solanum lycopersicum*	Greenhouse	Field soil	*Bacillus subtilis* *Ochrobactrum pseudogrignonense*	Root dip + soil drench		
Tobacco whitefly *Bemisia tabaci*	Pepper *Capsicum annuum*	Greenhouse	Field soil	Natural microbiomes	N/a	N/a	N/a
Tobacco whitefly *Bemisia tabaci*	Tomato *Solanum lycopersicum*	Greenhouse	Soil	*Pseudomonas (fluorescens) simiae* WCS417	Root dip		+
Tobacco whitefly *Bemisia tabaci*	Tomato *Solanum lycopersicum*	Greenhouse	Mixture of peat, soil conditioner, loam, mulch, vermiculite, perlite	*Bacillus subtilis* DN	Root dip + soil drench		

								No	N/a	de Laurentis et al., 2014	
					-/+			Confirmed in other experimental plants	N/a	Tétard-Jones et al., 20(	Effects depend on plant and aphid genotype; 6 plant genotypes, and 5 aphid genotypes. Of these, 2 aphid genotypes showed consistency over replicates. Genotype: CLO7 1 positive / 3 negative / 2 neutral. Genotype HF92: 3 positive/ 2 negative/ 1 neutral. Not the same plant genotypes.
	-/+							Confirmed in other experimental plants	N/a	Tétard-Jones et al., 20(	Effects depending on plant genotype: 48 lines: 51 % positive / 36 % negative / 13 % neutral
		No effect				-		No	N/a	Disi et al., 2018	
		No effect				-		No	N/a	Disi et al., 2018	
		No effect				-		No	N/a	Disi et al., 2018	
	No effect							No	N/a	De Vos et al., 2007	
			No effect					Yes	Negative	Kurm et al., 2018	
				No effect				Yes	N/a	Pineda et al. 2012	
N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	Yes	Gram-positive bacteria increased	Kim et al., 2016	
	No effect							No	N/a	Herman et al., 2008	
	+							Yes	Positive	Kim et al., 2016	
	-							No	N/a	Gandhi et al., 2006	
								Yes	N/a	Pangesti et al., 2015a	
		-					-	No	N/a	Commare et al., 2002	
		-					-	No	N/a	Commare et al., 2002	
							-	No	N/a	Commare et al., 2002	
							-	No	N/a	Commare et al., 2002	
		-					-	No	N/a	Saravanakumar et al., 2008	
							-	No	N/a	Saravanakumar et al., 2008	
		-					-	No	N/a	Saravanakumar et al., 2008	
							-	No	N/a	Saravanakumar et al., 2008	
		-					-	No	N/a	Saravanakumar et al., 2008	
							-	No	N/a	Saravanakumar et al., 2008	
	-/No effect							No	N/a	Murphy et al., 2000	Different developmental stages were counted; effects dependend on method of application, *Bacillus* species/strain and year
	-/No effect							No	N/a	Murphy et al., 2000	
	-/No effect							No	N/a	Murphy et al., 2000	
	-/No effect							No	N/a	Murphy et al., 2000	
							-	No	N/a	Zehnder et al., 1997	
	-							No	N/a	Zehnder et al., 2000	Results reported in text and figure conflicting
					No effect			No	N/a	Sripontan et al., 2014	
					-/+			No	N/a	Sripontan et al., 2014	No fertillizer = no effect Half fertilizer amount = positive Full fertlizer amount = negative
N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	Yes	Relative abundance was altered	Kong et al., 2016	
		+				No effect		Yes	No effect	Shavit et al., 2013	
	-							Yes	Unpublished	Valenzuela-Soto et al., 2010	

Beet armyworm *Spodoptera exigua*	Barrelclover *Medicago truncatula*	Greenhouse	Peat/soil conditioner	*Sinorhizobium meliloti*	Soil drench	No effect	
Cotton leafworm *Spodoptera littoralis*	White clover *Trifolium repens*	Greenhouse	Soil/sand	*Rhizobium leguminosarum*	Applied on seeds	No effect	
Green peach aphid *Myzus persicae*	White clover *Trifolium repens*	Greenhouse	Soil/sand	*Rhizobium leguminosarum*	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Field	Soil	*Bradyrhizobium japonicum*	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Field	Soil	*Bradyrhizobium japonicum* *Azospirillum brasilense*	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Field	Soil	*Bradyrhizobium japonicum* *Delftia* spp.	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Greenhouse	Soil	*Bradyrhizobium japonicum* *Delftia* spp.	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Greenhouse	Field soil	*Bradyrhizobium japonicum*	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Greenhouse	Field soil	*Bradyrhizobium japonicum* *Azospirillum brasilense*	Applied on seeds		
Soybean aphid *Aphis glycines*	Soybean *Glycine max*	Field	Soil	Natural microbiomes	Applied on seeds		

								Yes, nodules counted	Positive	Heath et al., 2011	
								Yes, nodules counted	Positive	Kempel et al., 2009	
	No effect							Yes, nodules counted	N/a	Kempel et al., 2009	
	-/No effect							Yes, nodules counted	Negative	Brunner et al., 2015	
	No effect							Yes, nodules counted	Negative	Brunner et al., 2015	
	-/No effect							Yes, nodules counted	Negative	Brunner et al., 2015	
	-							Yes, nodules counted	Negative	Brunner et al., 2015	
	No effect							Yes, nodules counted	Negative	Brunner et al., 2015	
	No effect							Yes, nodules counted	Negative	Brunner et al., 2015	
	+							Yes, nodules counted	N/a	Dean et al., 2009	

Chapter 3

Differential effects of the rhizobacterium *Pseudomonas simiae* on above- and belowground chewing insect herbivores

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Published in a revised version in Journal of Applied Entomology 145:250–260 (2021).

Abstract

Plant growth-promoting rhizobacteria (PGPR) can enhance plant growth and plant defense. *Via* plant-mediated effects PGPR have been reported to impact the performance of generalist leaf-chewing insects either negatively or positively. However, only a few insect species, mainly feeding on aboveground tissues, have thus far been investigated.

Here, we investigated how addition of rhizobacteria to plant soil affects the performance of three chewing insect herbivores, two leaf chewers and one root feeder. In a greenhouse experiment we grew white cabbage (*Brassica oleracea*) plants in soil supplemented with the rhizobacterium *Pseudomonas simiae* WCS417r. We investigated the consequences for larvae of the cabbage moth *Mamestra brassicae*, the diamondback moth *Plutella xylostella*, or the cabbage root fly *Delia radicum* after 5 weeks of plant growth. We recorded insect biomass, aboveground plant biomass, plant-defense marker gene expression levels and plant defense-related hormone concentrations.

Bacterial inoculation increased aboveground plant biomass in non-infested plants but not in infested plants. Rhizobacterial inoculation affected insect performance differently: on PGPR-inoculated plants *Plutella xylostella* biomass was lower, *Mamestra brassicae* biomass was similar, and *Delia radicum* biomass was higher than on control plants. Rhizobacterial inoculation increased the expression of the marker gene *LOX2* in *P. xylostella* infested plants. Levels of the hormones jasmonic acid, salicylic acid and abscisic acid were similar in inoculated and non-inoculated plants. Transcription levels of the plant defense marker gene showed upregulation of *PAL1* between inoculated and non-inoculated insect-free plants. We conclude that rhizobacterial inoculation has costs and benefits for cabbage production. The balance of these costs and benefits needs to be assessed in order to conclude on the value of inoculating this PGPR for cabbage production.

Keywords Cabbage root fly (*Delia radicum*), cabbage moth (*Mamestra brassicae*), diamondback moth (*Plutella xylostella)*, plant resistance, rhizobacteria (*Pseudomonas simiae* WCS417r), white cabbage (*Brassica oleracea*)

Introduction

Insect damage to crops is estimated to be five to twenty percent of global crop production (Deutsch *et al.*, 2018). Increasing agricultural output calls for effective insect pest control, which is at the same time environmentally sustainable. An innovative contribution to sustainable pest control is the use of beneficial soil microbes. Beneficial microbes can enhance plant growth and defense against a range of attackers (Kloepper *et al.*, 2004; Raaijmakers *et al.*, 2009; Pineda *et al.*, 2010; Pieterse *et al.*, 2014). A group of plant beneficial microbes are plant growth-promoting rhizobacteria (PGPR). Plants may gain benefits from the interaction with the bacteria, for example through increased photosynthesis (Zhang *et al.*, 2008) or increased nutrient supply (Pii *et al.*, 2015). There are also indirect effects on plant fitness, such as the exclusion of pathogenic bacteria (Massalha *et al.*, 2017) or antibiotic production by rhizobacteria (de Souza *et al.*, 2003). Moreover, PGPR may mediate induced systemic resistance towards attackers such as herbivorous insects and pathogenic microbes (Pieterse *et al.*, 1998; Pineda *et al.*, 2010; Pieterse *et al.*, 2014).

Rhizobacterial colonization primes plant defense against future attack: defense responses to attack are induced faster and stronger as a result of root colonization by PGPR (Conrath *et al.*, 2006; Pieterse *et al.*, 2014). Rhizobacterially induced systemic resistance (ISR) requires an intact jasmonic acid (JA) signaling pathway (Pieterse *et al.*, 2012). Generally, rhizobacteria-triggered ISR has adverse effects on insect herbivores, specifically on chewing insects that mainly trigger the JA defense signaling pathway (Pineda *et al.*, 2010; Berendsen *et al.*, 2012; Erb *et al.*, 2012b). Phloem-feeding insects that mainly induce the salicylic acid (SA) signaling pathway, are hypothesized to be less affected by rhizobacterial colonization. Nevertheless, there are examples where rhizobacterial colonization does not influence insect attackers (Disi *et al.*, 2018) or even favors insect performance (Boutard-Hunt *et al.*, 2009; Pineda *et al.*, 2012; Megali *et al.*, 2014).

The consequences for insect feeding on rhizobacteria-colonized plants may depend on several factors, such as the plant organ attacked. Plant defense can vary between belowground and aboveground tissues (Biere & Goverse, 2016). For example, in response to belowground herbivory, jasmonic acid levels are enhanced in roots, but not as much as JA levels in leaves in response to aboveground herbivory. This suggests that hormonal sensitivity in roots is higher than in aboveground tissues (Erb *et al.*, 2012a). Another difference between aboveground and belowground environments are the insects that feed in them. Root herbivores have different traits compared to aboveground herbivores,

such as longer life cycles and limited mobility (Johnson *et al.*, 2016). So far, the effect of PGPR on plant defense against belowground-feeding insects has received little attention.

Here, we investigated the effects of a PGPR on cabbage plants, in terms of plant growth and plant defense to three chewing insect herbivores, one root-feeding species and two leaf-feeders. The cabbage root fly *Delia radicum* is considered a pest insect herbivore on roots of cruciferous crops, mainly in northern Europe. The females lay eggs near the stem of the plant, and after hatching the larvae mine into the plant's main root. The larvae pupate in the soil. The diamondback moth *Plutella xylostella* is the most important pest herbivore of cruciferous crops and has a global distribution (Zalucki *et al.*, 2012). The first instar larvae are leaf miners, and the older larvae feed on the underside of the leaves with a preference for the younger leaves. The cabbage moth *Mamestra brassicae* feeds on several crop species and is a pest insect in Europe and Asia. The larvae feed first on the older leaves, but will later tunnel through the crop head, leaving behind frass that contributes to crop rotting.

We employed the rhizobacterium *Pseudomonas simiae* WCS417r, formerly *P. fluorescens* WCS417r, and the crucifer crop *Brassica oleracea* cv *capitata*. We addressed the following research questions: (i) How does *Pseudomonas simiae* WCS417r inoculation affect the performance of *Plutella xylostella, Mamestra brassicae* and *Delia radicum* larvae? (ii) How does *Pseudomonas simiae* WCS417r colonization and insect feeding affect plant defense responses? We hypothesized that rhizobacterial inoculation increases plant biomass and affect insect performance, which would support the use of these microbes in a sustainable agriculture.

Materials and Methods

Rhizobacterium Pseudomonas simiae WCS417r and plant growth conditions

The non-pathogenic epiphytic rhizobacterium *Pseudomonas simiae* WCS417r, a rifampicin-resistant strain, was grown on King's B (KB) medium agar plates containing 25 µg ml^{-1} rifampicin during 48 h at 28°C (Pieterse *et al.*, 1996). Prior to soil inoculation, a bacterial solution was made with sterilized 10 mM $MgSO_4$ and adjusted to a cell density of 1×10^9 colony forming units (CFU) ml^{-1} (OD_{660} = 1.0).

Seeds of white cabbage (*Brassica oleracea* cv. Christmas Drumhead, provided by the Centre for Genetic Resources, Wageningen, The Netherlands) were surface-sterilized with 80% ethanol for 1 minute, followed by 15 minutes in 1 % hypochlorite solution and washed three times with sterilized tap water. Seeds were incubated at 5°C for 3 days to synchronize germination, and sown on twice autoclaved (121°C, 20

minutes, 24 h in between treatments) soil (Horticoop b.v., Slingerland Potgrond) mixed 1:3 with Perlite (Agra-perlite, grain size 3). Either bacterial solution or 10 mM $MgSO_4$ solution was added to the soil at 50 ml kg^{-1}. After one week, plants were transplanted to 11 x 11 x 12 cm pots with soil that was treated with *P. simiae* as previously described or with control soil treated with sterilized 10 mM $MgSO_4$. Plants were watered twice per week or when needed, and 50 ml fertilizer Hyponex® was added once per week after transplanting. Plants were grown at 20 ± 2°C and 70 % RH in a greenhouse. Photoperiod was maintained at 16:8 h (light:dark) with additional lighting provided by halide bulbs (400 W) when photosynthetic active radiation (PAR) dropped below 400 µmol s^{-1} m^{-2}. The plants used in the experiments were 5 weeks old (Table **1**).

Table 1 Overview of the experimental design. *Pseudomonas simiae* is used as soil inoculant.

			Plant growth		**Number of individuals measured for biomass**	**Mean day *D. radicum* emergence PI**	**Gene exp. 24 h PI**	**Carbon-Nitrogen and Phyto-hormones analysis**
Insect treatment	**Inoculation**	**Plants**	**6 weeks**	**12 weeks**			**Pooled plants**	**Pooled plants**
Plutella xylostella	None	20	x		80		4	4
	P. simiae	20	x		76		4	4
Mamestra brassicae	None	20	x		82		4	4
	P. simiae	20	x		87		4	4
No insect	None	10	x				2	2
	P. simiae	10	x				2	2
Delia radicum	None	21		x	122	38	4	4
	P. simiae	20		x	89	39	4	4
No insect	None	10		x			2	2
	P. simiae	10		x			2	2

Insect rearing

Cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae), was caught in 2013 near Zeewolde, The Netherlands and reared at 22 ± 2°C, natural daylight, and fed on 1:1:1 mix of milk powder, sugar and yeast flakes. Larvae were reared on roots of Rutabaga, *Brassica napus*. Cabbage moth larvae *Mamestra brassicae* L. (Lepidoptera: Noctuidae) were reared on *Brassica oleracea* L. *gemmifera* cv. Cyrus at 22 ± 2°C, 40-50 % RH, 16L:8D photoperiod. Larvae of the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) were reared on *B. oleracea* L. *gemmifera* cv. Cyrus (22 ± 2°C, 40-50 % RH, 16L:8D photoperiod. Newly hatched larvae of all three species were used in the experiments.

Insect performance, plant growth measurements and plant sampling

Plants were covered with a nylon mesh bag. On each plant either 5 neonate larvae of *M. brassicae* or *P. xylostella* were placed. For the root herbivore *D. radicum,* 20 neonate larvae were added carefully to the exposed top segment of the roots. Aboveground insects were weighed on day 4 and day 10 post infestation (dpi) on a microbalance (CP2P, Sartorius AG, Germany) to the nearest 0.001 mg. Belowground insects emerging as adults from the soil in the pots were caught in the mesh bags and collected once every 24 h. Flies were then frozen and weighed on a microbalance (CP2P, Sartorius AG, Germany) to the nearest 0.001 mg. After insect removal, plants were harvested and their fresh weight was determined. Subsequently, plants were dried at 70°C for 48 h, and weighed individually to the nearest 0.1 mg (Mettler Toledo, Switzerland). For nutrient analysis the fifth leaf was collected, counted from the top. Leaves from four plants were pooled for infested plants, and from two plants for uninfested plants. The leaves were freeze-dried, ground in liquid nitrogen and weighed. Dried samples were stored at -20°C until analysis. Carbon and nitrogen content were assessed with a CHNS analyser (TruSpec CN determinator, LECO Corporation, MI, USA).

By the end of the experiment the *B. oleracea* roots were inspected to confirm the presence of *Pseudomonas* bacteria. Root material (1 g fresh biomass) was collected and shaken vigorously for 1 min in 9 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (425–600 µm, Sigma-Aldrich), and the solution was serially diluted. Dilutions were plated with 50 µl solution in duplicate onto KB agar medium supplemented with 25 µg ml^{-1} rifampicin, cyclohexamine 100 mg ml^{-1}, chloramphenicol 13 mg ml^{-1} and ampicillin 50 mg ml^{-1} to select for fluorescent *Pseudomonas* spp. (Pieterse *et al.*, 1996). The dilution plates were incubated for 48 h at 28°C.

Plant gene expression analysis

From the same batch of plants used for insect performance, we evaluated gene expression of the JA/ET associated *LIPOXYGENASE-2* (*LOX2*), transcription factor (*MYC2*), and SA associated genes *PHENYLALANINE AMMONIA-LYASE-1* (*PAL1*) and *PATHOGENESIS-RELATED PROTEIN-1* (*PR1*) in leaves. One leaf disk (1 cm diameter) was collected 24 h after infestation. For *M. brassicae* or *P. xylostella* infested plants the first fully expanded herbivore damaged leaf was sampled. For *D. radicum* infested plants and control plants, the fourth leaf from the top was sampled. Tissue was immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Leaf disks from four plants were pooled for each insect treatment and from two plants for the control treatment

without insects. Leaf samples were ground with pestle and mortar in liquid nitrogen.

Total RNA was isolated with an RNA extraction kit (Isolate II RNA Plant Kit, Bioline), according to the manufacturer's protocol. Synthesis of cDNA was carried out with a cDNA synthesis kit (SensiFAST, Bioline) following the manufacturer's instructions and diluted five times. Stock primers were diluted four times. Efficiency of each primer was determined before qRT-PCR analysis (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). For the full primer list, see Supplement Table **S1**. A Bio-rad 1000 machine was used to carry out q-PCR. Reaction mixtures (25 µl) contained 10 µl of SYBR Green qPCR master mix (Bio-rad), cDNA and 5 µl of each primer. The thermocycle parameters were as following: initial polymerase activation, 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 60 s at 57°C and 30 s at 72°C. Conditions were determined by temperature gradient testing for all primers and a mixture of eight random samples cDNA from the experiment. From six reference genes (*Act-2, Btub, EF1a, GAPDH, PER4, SAR1a*), *SAR1a* and *Act-2* were selected as optimal reference genes. Relative gene expression was calculated considering primer efficiency with the software qBase+ 3.1 (Biogazelle, Zwijnaarde, Belgium), through the CNRQ (Calibrated Normalized Relative Quantity) method.

Analysis of phytohormones jasmonic acid, salicylic acid and abscisic acid

From the same samples that were used for gene expression, a portion was lyophilized (Snijders type 2040 lyophylizer, Tilburg, The Netherlands) and extracted with methanol. Phytohormone analysis was performed on an Agilent 1200 series HPLC system (Agilent Technologies) coupled to a tandem mass spectrometer (Vadassery *et al.*, 2012), with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. Total concentration of jasmonates (JAs) was calculated by summation of the concentrations of JA, JA-Ile, cis-OPDA, OH-JA, OH-JA-Ile and COOH-JA-Ile.

Statistical analysis

To check normality and homogeneity of the data we used Shapiro-Wilk's and Levene's tests, and inspecting residuals through visualisation using qq-plots and Cullen and Frey graphs. Insect performance data was analysed with a Generalized Linear Mixed Model with plant as a random factor. Plant biomass, gene expression, phytohormone and plant carbon and nitrogen data was analysed with Student's *t*-test or Generalized Linear Model depending on normality and homogeneity of the data, with $\alpha = 0.05$. If suitable, post hoc Tukey tests were run with a maximum

likelihood fit (Laplace Approximation). The statistical tests were carried out with RStudio version 1.1.423 (R Core Team, 2018) using packages car (Fox & Weisberg, 2019), lmtest (Zeileis & Hothorn, 2002), readxl, ggplot2, fitdistrplus and lme4 (Bates *et al.*, 2015).

Results

Effect of P. simiae soil inoculation on P. xylostella larval biomass and plant traits

Rhizobacterial soil inoculation significantly decreased the body mass of *P. xylostella* caterpillars by 20 % (χ^2 = 7.25, df = 1, p = 0.007) compared to the control after 10 days of insect feeding (Fig. **1**). Shoot dry weight, leaf carbon and nitrogen content of plants infested by *P. xylostella* were not affected by rhizobacterial soil inoculation (shoot dry weight: χ^2 = 0.08, df = 1, p = 0.78; carbon content: t = -1.01, df = 8, p = 0.34; nitrogen content: t = -0.73, df = 8, p = 0.49). We explored possible phytochemical mechanisms for the reduced larval growth by examining defense-related plant traits. The transcript level of the plant defense marker gene *LOX2* was higher in leaves of plants grown in *P. simiae* inoculated soil compared to leaves of control plants after 24 h of insect infestation; transcript levels for the other three genes were not affected by *P. simiae* inoculation (*LOX2*: χ^2 = 13.07, df = 1, p < 0.001; *MYC2*: χ^2 = 0.24, df = 1, p = 0.63; *PAL1*: χ^2 = 0.46, df = 1, p = 0.50; *PR1*: χ^2 = 0.45, df = 1, p = 0.50). The concentrations of the phytohormones JA, SA and ABA in *P. xylostella*-infested leaves were not affected by soil inoculation with *P. simiae* (JA: t = 0.66, df = 8, p = 0.52; total jasmonates: χ^2 = 1.07, df = 1, p = 0.30; SA: χ^2 = 1.47, df = 1, p = 0.23; ABA: t = 0.45, df = 8, p = 0.66).

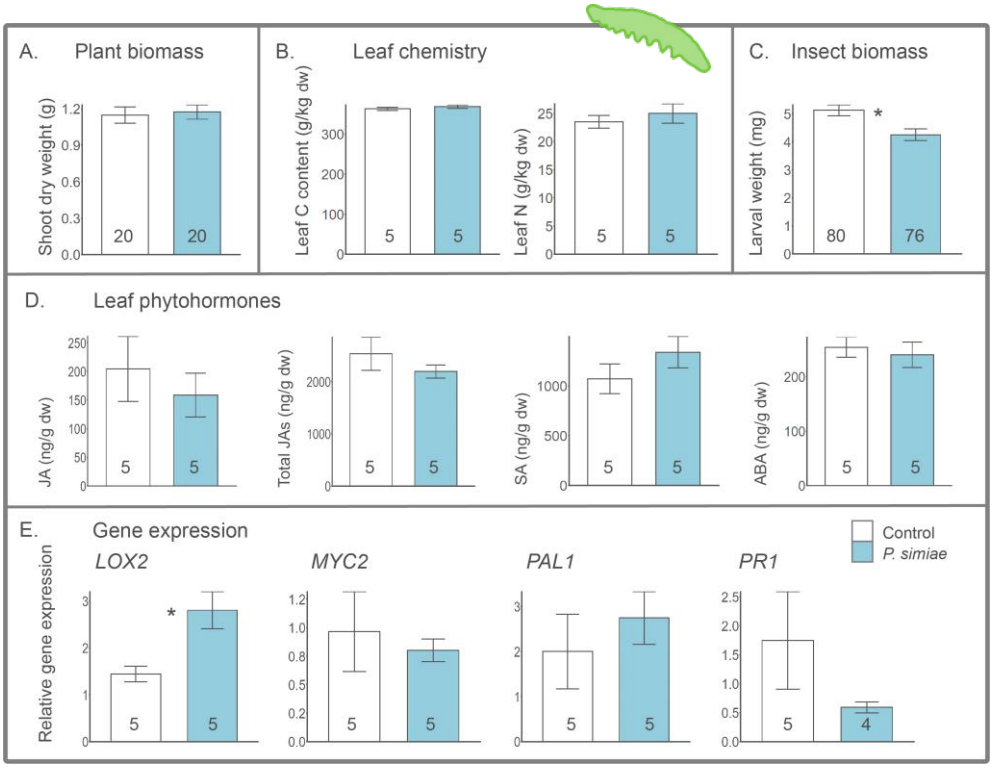

Figure 1 Effects of *Pseudomonas simiae* soil inoculation on *Plutella xylostella* growth performance and plant parameters. **A.** Shoot dry weight of *P. xylostella*-infested cabbage plants growing in inoculated or non-inoculated soil. **B.** Carbon and nitrogen content of leaves with *P. xylostella* feeding on inoculated or non-inoculated cabbage plants in g kg^{-1} dry weight. **C.** *Plutella xylostella* larval biomass 10 days post infestation feeding on inoculated or non-inoculated cabbage plants. **D.** Concentrations of the plant hormones jasmonic acid (JA) and total jasmonates (JAs), salicylic acid (SA) and abscisic acid (ABA) in leaves of *P. xylostella*-infested plants growing in inoculated or non-inoculated soil in ng g^{-1} dry weight. **E.** Plant defense marker gene expression in leaves of *P. xylostella*-infested plants growing in inoculated or non-inoculated soil relative to expression of housekeeping genes. Bars show mean ± SE; numbers in bars are number of replicates per treatment.

Effect of P. simiae soil inoculation on M. brassicae larval biomass and plant traits

Rhizobacterial soil inoculation did neither influence the body mass of *M. brassicae* caterpillars after 10 days of feeding (χ^2 = 0.25, df = 1, p = 0.61; Fig. **2**), nor plant shoot dry weight, carbon or nitrogen leaf content (shoot dry weight: χ^2 = 0.08, df = 1, p = 0.77; carbon content: t = -0.86, df = 4.44, p = 0.43; nitrogen content: t = -0.35, df = 8, p = 0.74) of *M. brassicae*-infested plants. Rhizobacterial inoculation did not affect phytohormone levels of JA, total jasmonates, SA or ABA in leaves (JA: t = 0.73, df = 8, p = 0.48; total jasmonates: t = 1.16, df = 8, p = 0.28; SA: χ^2 = 0.78, df = 1, p = 0.38; ABA: χ^2 < 0.01, df = 1, p = 0.93) of plants infested by *M. brassicae*. Finally, rhizobacterial soil inoculation affected transcript levels of marker gene *MYC2*, whereas the levels of the three other marker genes were not affected in leaves after 24 h of caterpillar feeding (*LOX2*: χ^2 = 0.05, df = 1, p = 0.83; *MYC2*: χ^2 = 6.02, df = 1, p = 0.014; *PAL1*: χ^2 < 0.01, df = 1, p = 0.96; *PR1*: χ^2 = 2.65, df = 1, p = 0.10).

Effect of P. simiae soil inoculation on D. radicum adult biomass and plant traits

Rhizobacterial inoculation resulted in increased adult weight of *D. radicum* (χ^2 = 6.85, df = 1, p = 0.008; Fig. **3**). Development time until insect adult emergence was similar for inoculated and non-inoculated plants (non-inoculated: median = 37 days post infestation DPI, 3rd quartile = 39 DPI; inoculated: median = 38 DPI, 3rd quartile = 39 DPI; GLMM: χ^2 = 2.07, df = 1, p = 0.15; data not shown). Shoot dry weight of *D. radicum*-infested plants was similar for inoculated and non-inoculated plants (t = -1.61, df = 39, p = 0.12). Soil inoculation did not influence leaf carbon or nitrogen content (carbon content: t = -1.23, df = 8, p = 0.25; nitrogen content: t = -1.62, df = 8, p = 0.14) of plants infested by *D. radicum*. Insect infestation and rhizobacterial inoculation did not affect the foliar concentrations of the phytohormones JA, total jasmonates or ABA, but did have an effect on SA levels (JA: t = -0.31, df = 8, p = 0.76; total jasmonates: χ^2 = 0.49, df = 1, p = 0.48; SA: χ^2 = 5.34, df = 1, p = 0.021; ABA: χ^2 = 2.46, df = 1, p = 0.12) or defense marker gene expression in leaves of *D. radicum*-infested plants after 24 h of herbivory (*LOX2*: χ^2 < 0.01, df = 1, p = 0.97; *MYC2*: χ^2 = 0.47, df = 1, p = 0.49; *PAL1*: χ^2 = 0.96, df = 1, p = 0.32; *PR1*: χ^2 = 0.16, df = 1, p = 0.67).

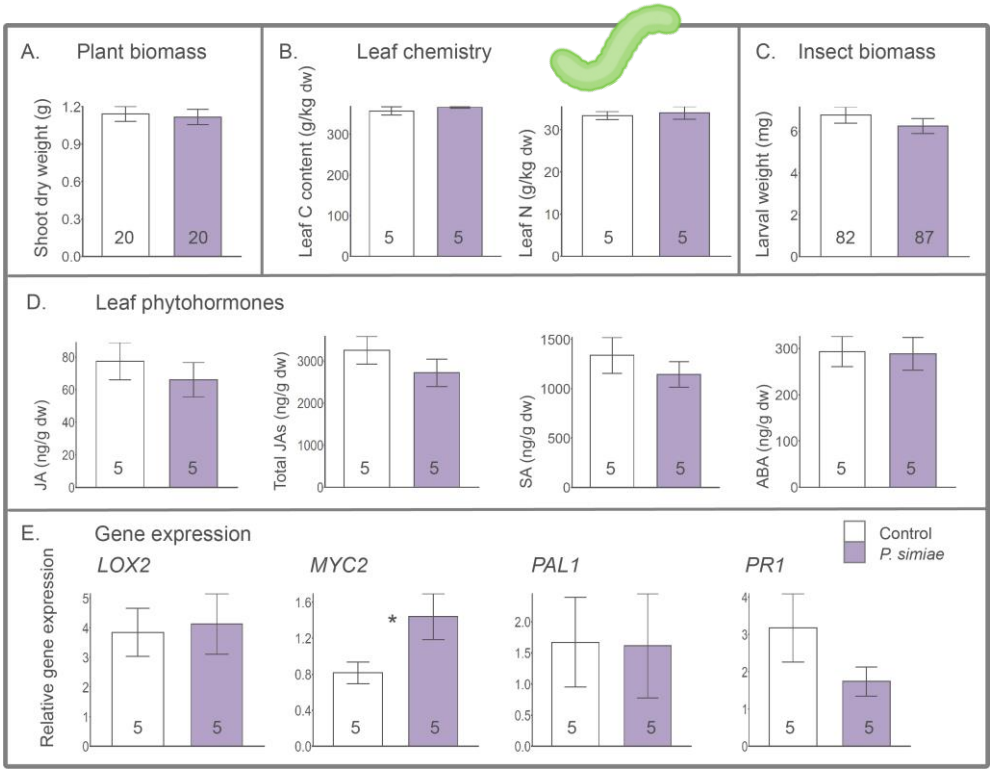

Figure 2 Effects of *Pseudomonas simiae* inoculation on *Mamestra brassicae* growth performance and plant parameters. **A.** Shoot dry weight of *M. brassicae*-infested cabbage plants growing in inoculated or non-inoculated soil. **B.** Carbon and nitrogen content in leaves with *M. brassicae* feeding on inoculated or non-inoculated cabbage plants in g kg^{-1} dry weight. **C.** *Mamestra brassicae* larval biomass 10 days post infestation feeding on inoculated or non-inoculated cabbage plants. **D.** Concentrations of the plant hormones jasmonic acid (JA) and total jasmonates (JAs), salicylic acid (SA) and abscisic acid (ABA) in leaves of *M. brassicae*-infested plants growing in inoculated or non-inoculated soil in ng g^{-1} dry weight. **E.** Plant defense marker gene expression in leaves of *M. brassicae*-infested cabbage plants growing in inoculated or non-inoculated soil relative to expression of housekeeping genes. Bars show mean ± SE; numbers in bars are number of replicates per treatment; '*' indicate significantly different mean values between treatments.

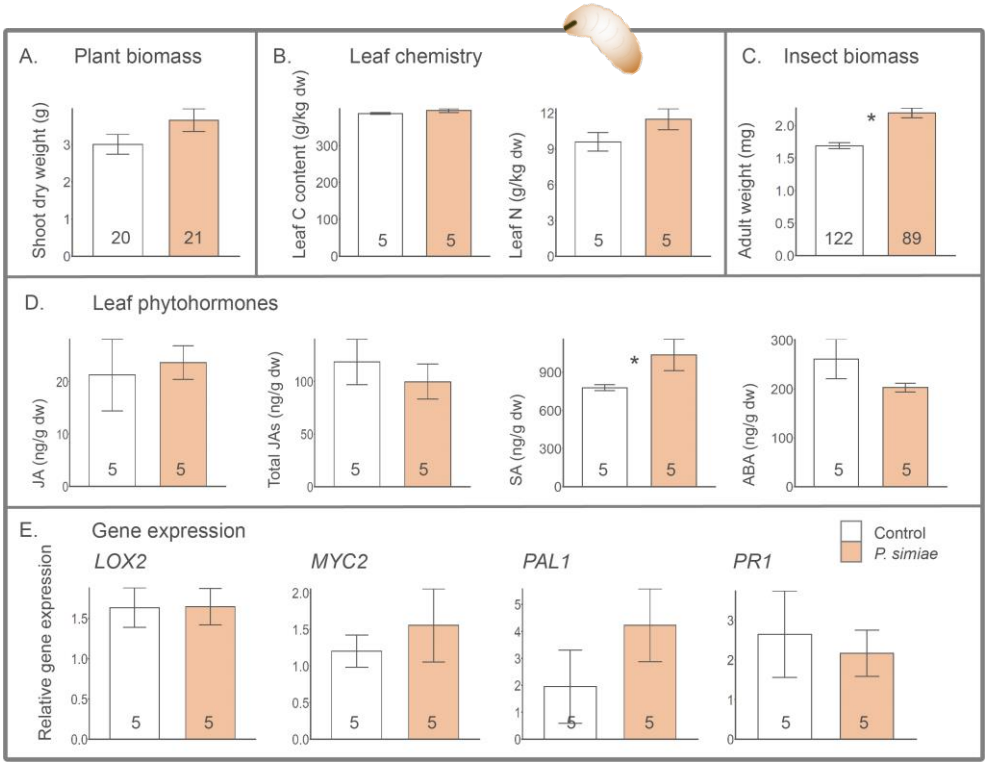

Figure 3 Effects of *Pseudomonas simiae* inoculation on *Delia radicum* growth performance and plant parameters. **A.** Shoot dry weight of *D. radicum*-infested cabbage plants growing in inoculated or non-inoculated soil. **B.** Carbon and nitrogen content in leaves with *D. radicum* feeding on inoculated or non-inoculated cabbage plants in g kg^{-1} dry weight. **C.** *Delia radicum* adult weight by emergence feeding on inoculated or non-inoculated cabbage plants. **D.** Concentrations of the plant hormones jasmonic acid (JA) and total jasmonates (JAs), salicylic acid (SA) and abscisic acid (ABA) in leaves of *D. radicum*-infested plants growing in inoculated or non-inoculated soil in ng g^{-1} dry weight. **E.** Plant defense marker gene expression in leaves of *D. radicum*-infested cabbage plants growing in inoculated or non-inoculated soil relative to expression of housekeeping genes. Bars show mean ± SE; numbers in bars are number of replicates per treatment; '*' indicate significantly different mean values between treatments.

Effect of P. simiae soil inoculation on traits of uninfested plants

The addition of *P. simiae* to the soil increased shoot dry weight of uninfested cabbage plants after six weeks of exposure by on average 33% ($t = -2.99$, $df = 18$, $p = 0.008$; Fig. **4**), whereas carbon or nitrogen content of leaves were not affected (carbon content: $t = -0.79$, $df = 7$, $p = 0.44$; nitrogen content: $t = 0.83$, $df = 7$, $p = 0.44$). Foliar concentrations of SA were higher and concentrations of ABA lower for plants inoculated with bacteria compared to control plants, whereas JA concentration was unaffected by bacterial inoculation (JA: $t = -0.26$, $df = 7$, $p = 0.80$; total jasmonates: $\chi^2 = 0.82$, $df = 1$, $p = 0.37$; SA: $t = -2.64$, $df = 7$, $p = 0.033$; ABA: $\chi^2 = 13.3$, $df = 1$, $p < 0.001$). Bacterial inoculation resulted in lower expression level of the marker gene PAL1 in leaves, whereas the expression levels of PR1, LOX2, and MYC2 were unaffected (LOX2: $\chi^2 = 3.76$, $df = 1$, $p = 0.053$; *MYC2*: $\chi^2 = 0.26$, $df = 1$, $p = 0.61$; *PAL1*: $\chi^2 = 7.07$, $df = 1$, $p < 0.001$; *PR1*: $\chi^2 = 0.01$, $df = 1$, $p = 0.91$).

For 12-week-old plants, rhizobacterial inoculation did neither affect shoot dry weight ($t = -0.75$, $df = 18$, $p = 0.46$; Fig. **5**) nor foliar carbon or nitrogen content (carbon: $t = -1.80$, $df = 7$, $p = 0.11$; nitrogen: $\chi^2 = 1.31$, $df = 1$, $p = 0.25$), nor foliar levels of JA, SA and ABA (JA: $t = 0.55$, $df = 7$, $p = 0.60$; total jasmonates: $\chi^2 = 0.52$, $df = 1$, $p = 0.47$; SA: $\chi^2 = 1.61$, $df = 1$, $p = 0.20$; ABA: $\chi^2 = 0.43$, $df = 1$, $p = 0.51$).

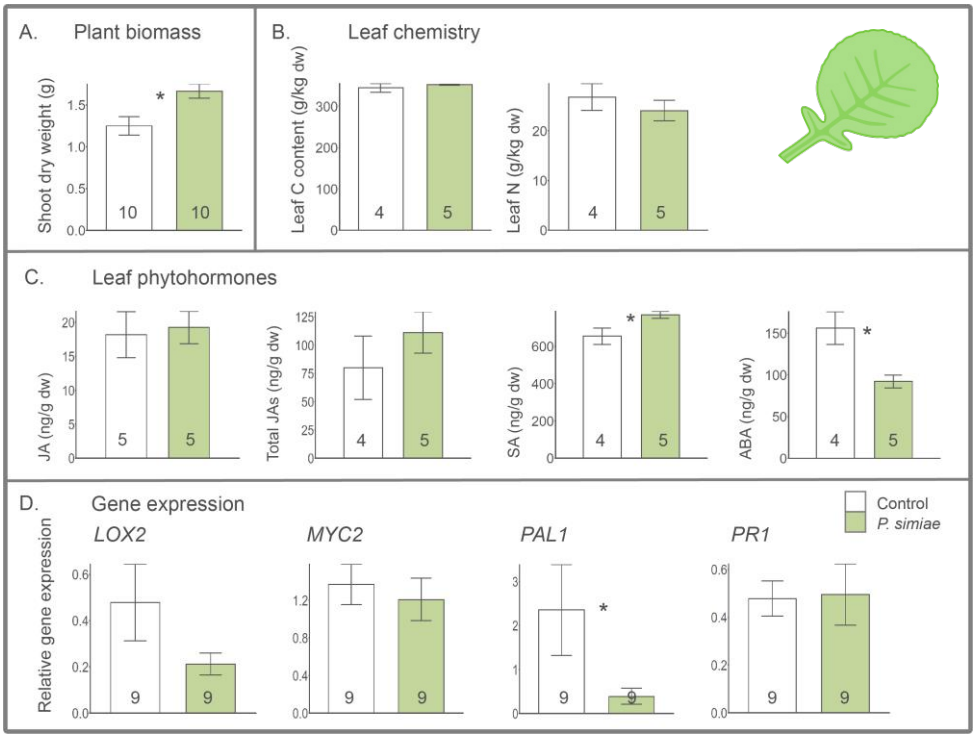

Figure 4 Effects of *Pseudomonas simiae* inoculation on 6-week-old cabbage plants. **A.** Shoot dry weight of cabbage plants growing in inoculated or non-inoculated soil. **B.** Carbon and nitrogen content in leaves of cabbage plants growing in inoculated or non-inoculated soil in g kg^{-1} dry weight. **C.** Concentrations of the plant hormones jasmonic acid (JA) and total jasmonates (JAs), salicylic acid (SA) and abscisic acid (ABA) in leaves of cabbage plants growing in inoculated or non-inoculated soil in ng g^{-1} dry weight. **D.** Plant defense marker gene expression in leaves of cabbage plants growing in inoculated or non-inoculated soil relative to expression of housekeeping genes. Bars show mean ± SE; '*' indicate significantly different mean values between treatments.

Re-isolation of Pseudomonas from plant rhizosphere

Pseudomonas bacterial concentration was higher in inoculated soil than in non-inoculated soil after both 6 weeks and 12 weeks of plant growth on agar media selective for *Pseudomonas*. The control soil contained on average 2.7×10^{6} *Pseudomonas* bacterial CFU g^{-1} of rhizosphere after 6 weeks. The inoculated soil contained approximately 76 times more, *i.e.* on average 2.05×10^{8} CFU g^{-1} of rhizosphere. After 12 weeks of plant growth, control soil had on average 5.74×10^{6} CFU g^{-1}, and inoculated rhizosphere soil had 2.5×10^{7} CFU g^{-1}.

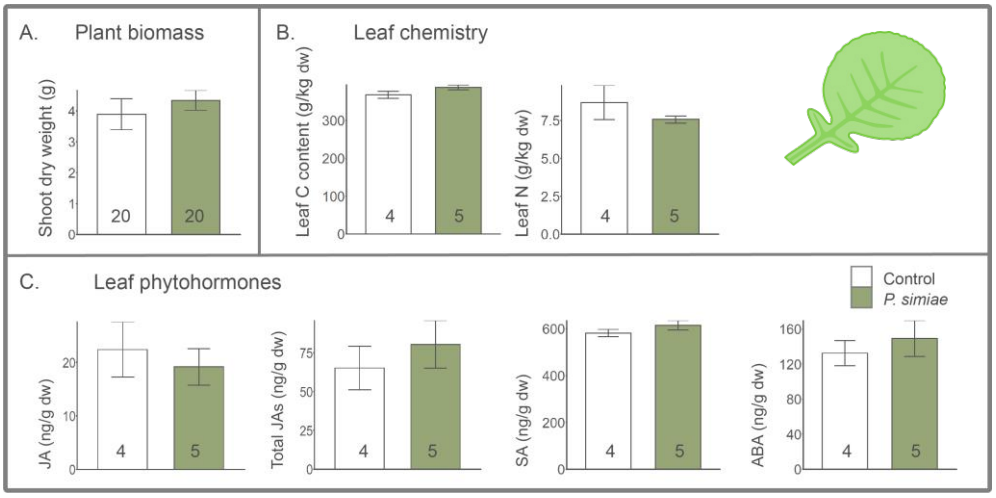

Figure 5 Effects of *Pseudomonas simiae* inoculation on 12-week-old cabbage plants. **A.** Shoot dry weight of cabbage plants growing in inoculated or non-inoculated soil. **B.** Carbon and nitrogen content in leaves of cabbage plants growing in inoculated or non-inoculated soil in g kg^{-1} dry weight. **C.** Concentrations of the plant hormones jasmonic (JA) and total jasmonates (JAs), salicylic acid (SA) and abscisic acid (ABA) in leaves ng g^{-1} dry weight. Bars show mean ± SE; numbers in bars are number of replicates per treatment; '*' indicate significantly different mean values between treatments.

Discussion

Our study shows that application of *Pseudomonas simiae* WCS417r differentially influences the performance of three chewing insect herbivores. Insect biomass data for *P. xylostella* showed decreased growth if cabbage plants were colonized by *P. simiae* WCS417r, suggesting an induced systemic plant defense response. This suggestion is supported by higher transcript levels of the defense marker gene *LOX2* in *P. xylostella*-infested leaves of rhizobacteria-inoculated plants. *Plutella xylostella* feeding has been previously shown to upregulate *LOX2* as part of plant defense in cabbage (Li *et al.*, 2016a). Upregulation of plant defense marker genes has previously been reported in conjunction with *P. simiae*-treatment in *A. thaliana* plants (Pangesti *et al.*, 2016). Concentrations of jasmonic acid (JA), salicylic acid (SA) or abscisic acid (ABA) in *P. xylostella*-infested leaves were not affected by rhizobacterial inoculation.

Rhizobacterial soil inoculation did not affect biomass of *M. brassicae* larvae, contrary to our expectation. Previous work shows that upregulation of *LOX2* is part of plant defense against *M. brassicae* feeding in cabbage (Pangesti *et al.*, 2015a; Li *et al.*, 2016a). In our study, neither *M. brassicae*-induced transcription of *LOX2*, nor jasmonate production nor JA-dependent transcription of *MYC2* were affected by rhizobacterial inoculation. This may explain why rhizobacteria did not affect *M. brassicae* weight. Studies on other plants show various effects of *P. simiae* inoculation on *M. brassicae* performance. When feeding on *A. thaliana* plants, *P. simiae* WCS417r colonization can both negatively and positively affect *M. brassicae* larvae, depending on soil nutrient concentration and drought stress (Pangesti *et al.*, 2015a; Fernández de Bobadilla *et al.*, 2017). Addition of fertilizer in our study may have offset potential negative effects of *P. simiae* on *M. brassicae* growth.

Delia radicum performed better on plants in rhizobacteria-treated soil compared to *D. radicum* on control plants. The effects of rhizobacterial inoculation of plants on *D. radicum* has not been studied so far. However, root microbial community structure is altered when *D. radicum* is feeding on oilseed rape (*Brassica napus*) (Ourry *et al.*, 2018). Additionally, another belowground chewer, the beetle *Diabrotica speciosa* Germar (Coleoptera: Chrysomelidae), is negatively affected by rhizobacterial inoculation of *Azospirillum brasilense* in maize (Santos *et al.*, 2014).

We propose three non-mutually exclusive mechanisms that may explain the increased *D. radicum* biomass. First, cabbage root biomass may have increased due to rhizobacterial inoculation, thereby increasing food availability to *D. radicum* larvae. How cabbage root biomass is affected by *P. simiae* is not yet known, although in the related plant *A.*

thaliana, *P. simiae* WCS417r increased root and shoot biomass (Pangesti *et al.*, 2017). Second, root production of secondary metabolites such as glucosinolates may be affected by PGPR colonization. PGPR inoculation may reduce glucosinolate levels in *A. thaliana* roots when colonized by *Kosakonia radicincitans* rhizobacteria (Witzel *et al.*, 2017). However, glucosinolate levels did not influence *D. radicum* performance in five wild cabbage populations (van Geem *et al.*, 2015). Yet, glucosinolates stimulate oviposition by *D. radicum* (Roessingh *et al.*, 1992), but it is not known whether the behavior of *D. radicum* larvae is affected by glucosinolate levels. A third explanation may be found in direct interactions between the PGPR and the insect. Compounds secreted by *P. simiae* may either act as feeding stimulants, or interact with insect gut microbes to increase nutrient acquisition. The gut microbiome of *D. radicum* larvae may contain PGPR. For example the PGPR strain *Pseudomonas* sp. PRGB06 has been found in the gut of *P. xylostella* (Indiragandhi *et al.*, 2008), but has yet to be found in belowground feeders. Further research is needed to determine whether *P. simiae* WCS417r stimulates feeding by *D. radicum*.

Uninfested rhizobacteria-inoculated plants exhibited downregulated expression of the marker gene *PAL1,* whereas expression of JA-associated marker genes was similar to that in uninfested control plants. Previous studies showed an upregulation of JA-related marker genes in *P. simiae* colonized *A. thaliana* plants (Pangesti *et al.*, 2016).

We found that rhizobacterial inoculation increased plant biomass, a result in line with previous research. Our results present the first report of cabbage growth promotion in response to colonization by *P. simiae* WCS417r. Cabbage plants have previously been shown to respond to rhizobacterial colonization by increased growth; several bacterial species increase plant growth (Turan *et al.*, 2014). Plant growth promotion by *P. simiae* WCS417r has been shown in other plant species such as grapevine, radish and banana (Berendsen *et al.*, 2015). Yet, for some plant species this strain did not promote growth, such as tobacco (van Loon *et al.*, 2008). Hence, the strain possesses specificity to plant species. This study's observed growth promoting effect was lacking in herbivore-infested plants. This may be explained by increase of plant defense in infested plants. According to the hypothesis of a growth-defense trade-off, plant resources will be distributed to either expansion or protection, where one will be adjusted for the other. An increase of defense in the experimental plants would thus be expected to result in lowered plant growth.

In conclusion, growth and defense of cabbage plants can benefit from the beneficial rhizobacterium *P. simiae* WCS417r. Interestingly, we

found a positive effect on the performance of the root herbivore *D. radicum* whereas for other chewing root herbivores negative effects were recorded. Hence, promotion of plant growth and improving resistance to chewing insect herbivores through soil inoculation with rhizobacteria is possible, yet insect performance can be differentially affected. Thus, the addition of this rhizobacterium has costs and benefits in terms of plant fitness. The relative effect sizes of these costs and benefits need to be assessed in order to assess the value of this PGPR for crop production.

Acknowledgments

The authors greatly appreciate support by the insect rearing staff of the Laboratory of Entomology for providing the insects used in this study. We would like to thank Michael Reichelt for technical assistance with phytohormone analysis and critical reading of the manuscript. This work was funded by Wageningen University and private funding.

Author Contribution

JF conducted the research and wrote the manuscript with input of AP, JG, MD and JJAvL. All authors read and approved the manuscript.

Supplemental information

Table S1 Sequences of primers used in quantitative RT-PCR analyses.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')
LOX2	GCCATTGAGTTGACTCGTCC	GGATGCATGGCACTTAGTTGT
MYC2	GGCTGGACCTACGCTATATTCTGG	AGAAAAACCACTCCGTATCCGT
PR1	GTCAACGAGAAGGCTAACTATAACTACG	TTACACTTGCTTTGCCACATCC
PAL1	TCGCTATGGCTTCTTACTGCTCTG	GAGGTCTTACGAGATGAGATGATGCC
*SAR1*a	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT
Act-2	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG

Chapter 4

Does seed treatment with *Pseudomonas simiae* rhizobacteria promote cabbage plant growth and insect herbivore performance?

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Abstract

Crop resistance against insects can be enhanced by root-associated bacteria. However, the application of such beneficial bacteria in agricultural systems poses challenges: when and how to apply them? The bio-priming method may be a solution, where seeds are hydrated together with beneficial rhizobacteria before sowing. Here, we investigated the biopriming method to explore plant resistance to herbivorous insects, through bacterizing *Brassica oleracea* seeds using the rhizobacterium *Pseudomonas simiae* WCS417r. We measured plant biomass and insect herbivore performance in greenhouse experiments. Cabbage plants were infested with the cabbage moth *Mamestra brassicae,* the diamondback moth *Plutella xylostella*, or the cabbage root fly *Delia radicum.*

We found that seed bacterization did not affect *B. oleracea* biomass. Biomass of *M. brassicae* and *P. xylostella* larvae was not affected. Data for biomass of *D. radicum* were inconclusive. In the first experiment, *D. radicum* biomass was not affected by seed bacterization. In contrast, the second experiment showed a positive effect from seed bacterization on *D. radicum* biomass. We conclude that seed bacterization as applied here did not result in enhanced plant resistance for the plant-rhizobacterium interaction studied. Overall, the field of seed biopriming needs standardization to optimize factors for colonization success to ensure yield increase.

Keywords Plant growth promoting bacteria, seed bio-priming, *Delia radicum*, *Mamestra brassicae*, *Plutella xylostella*, *Pseudomonas simiae* WCS417r.

Introduction

Environmental and health concerns are incentives for a reduction in pesticide usage in agriculture: sustainable crop protection strategies are urgently needed (Velten *et al.*, 2015; Sanchez-Bayo & Wyckhuys, 2019). A strategy that has emerged over the past decade is the application of plant-growth promoting rhizobacteria (PGPR) that have been shown to enhance plant resistance to microbial and insect attackers (Conrath *et al.*, 2006; Pineda *et al.*, 2010; Pieterse *et al.*, 2014; Rasmann *et al.*, 2017). Employing such beneficial bacteria for sustainable crop production brings a set of implementation challenges. For example, when and how to apply the beneficial microbes?

Adding microbes to the seeds can solve both the how and when to apply microbes for optimal crop production and resistance. An interesting microbe delivery application is the seed bio-priming method (Taylor *et al.*, 1998; O'Callaghan, 2016). This method is a sub-category of the wider concept of seed priming, where seed metabolism is initially activated through hydration (soaking seeds in fluid), followed by drying to halt seed germination. In the seed-biopriming method the seeds are bacterized by adding living beneficial bacteria in the hydration phase (O'Callaghan, 2016; Rocha *et al.*, 2019). Seed biopriming was shown to deliver sufficient amounts of bacteria to enhance plant growth. For example, growth of *Brassica napus* plants was enhanced upon seed bio-priming with *Pseudomonas chlororaphis* (Abuamsha *et al.*, 2011b).

Seed bio-priming can not only promote plant growth, but was shown to also promote plant resistance in crops against plant pathogens (Abuamsha *et al.*, 2011a; Sekar *et al.*, 2018; Jayapala *et al.*, 2019; Singh *et al.*, 2020). However, seed biopriming against plant attackers such as insects has rarely been explored. Here, we investigated the effects of seed treatment with the beneficial rhizobacterium *Pseudomonas simiae* WCS417r on growth and resistance to insect herbivores in cabbage, *Brassica oleracea*. We infested the plants with the herbivorous insect larvae of the cabbage moth *Mamestra brassicae*, diamondback moth *Plutella xylostella* and cabbage root fly *Delia radicum*, and measured plant growth and insect biomass increase as a proxy for plant resistance.

Materials and methods

Multiplication of rhizobacterium Pseudomonas simiae WCS417r and preparation of seed biopriming solution

The rhizobacterium *Pseudomonas simiae* WCS417r (formerly *P. fluorescens* WCS417r (Berendsen *et al.*, 2015)) was grown on King's B (KB) medium agar plates with added rifampicin (25 µg ml^{-1}), at 28°C for 48 h (Pieterse *et al.*, 1996). Cells were harvested and diluted in 10 mM

$MgSO_4$, and the solution was adjusted to a cell density of 1×10^9 colony forming units (CFU) ml^{-1} (OD_{660} = 1.0) with a spectrophotometer (Biorad SmartSpec™ 3000).

Insect rearing

The cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae) was reared in a climate cabinet (22 ± 2 °C, 40-60% RH, 16 L:8 D) and fed 1:1:1 mix of milk powder, sugar and yeast flakes. Larvae were reared on roots of rutabaga (*Brassica napus* L.). The cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) was reared on *Brassica oleracea* L. *gemmifera* cv. Cyrus in a climate room (22 ± 2 °C, 40-50 % RH, 16 L:8 D). The diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) was reared on *Brassica oleracea* L. *gemmifera* cv. Cyrus in a climate room (22 ± 2 °C, 40-50 % RH, 16 L:8 D). Newly hatched larvae of *M. brassicae* and *D. radicum*, and L2 larvae of *P. xylostella* were used in the experiments.

Seed bacterization and plant growing conditions

To bacterize white cabbage *B. oleracea* seeds (*Brassica oleracea* cv. Christmas Drumhead, provided by the Centre for Genetic Resources, Wageningen, The Netherlands), we first sterilized the seeds by dipping them in 80% ethanol for 2 min, followed by 15 min in 1% hypochlorite and 3 times in sterilized water using a tea-infuser. The water-soaked sterilized seeds (control) were soaked in sterilized 10 mM $MgSO_4$, whereas bacterized seeds were soaked in rhizobacterial solution, prepared as described above. Seeds were soaked in the dark in a rotator at 80 r.p.m. at room temperature for 4 h. The seeds were then dried in a desiccator in the dark for 24 h. To inspect bacterization, we crushed nine bacterized seeds and six water-soaked seeds in pools of three, in sterilized 10 mM $MgSO_4$. The solution was plated in duplicate on KB medium agar supplemented with 25 µg ml^{-1} rifampicin, 100 mg ml^{-1} cyclohexamine, 13 mg ml^{-1} chloramphenicol, and 50 mg ml^{-1} ampicillin. After incubating for two days we counted the CFU, and found a bacterial load of 7900 ± 760 CFU/seed (mean ± SE).

The experiment was done twice. In the first experiment we measured germination percentage and plant growth after 6 weeks for four seed treatments: 'no treatment', 'sterilized seeds', 'water-soaked seeds' and 'bacterized seeds'. Seed sterilization, water soak and bacterial treatment were performed as described above. Seeds were germinated on wet filter paper in a greenhouse, and seed germination was scored daily at the same time. In parallel, ten additional seeds from each of the four seed treatments and fifty additional water-soaked and bacterized

seeds were sown. The seeds were sown on twice autoclaved (121°C for 20 min, and 24 h in between autoclaving) soil (Horticoop b.v., Slingerland Potgrond) in a sowing tray.

After one week of growing, seedlings were transplanted to 11 x 11 x 12 cm pots. The potting soil was mixed 1:3 with Perlite (Agra-perlite, RHP, grain 3) and sterilized as described above before usage. Plants were watered three times per week until the soil was completely moist. Fertilization (50 ml Hyponex ®) was added after transplant, once during the first week, and then twice per week. Plants and seeds were germinated/grown in a greenhouse with settings 20 ± 2°C with 70 % RH. Photoperiod was maintained at 16 L:8 D h with additional lighting provided by halide bulbs (400 W) when photosynthetic active radiation (PAR) dropped below 400 µmol s^{-1} m^{-2}.

Plant and insect performance

After six weeks of plant growth, the 40 plants grown from the four seed treatments were harvested. Plant dry shoot biomass was determined after drying the aboveground tissue at 70°C for minimally 48 h, and weighed to the nearest 0.1 mg (Mettler Toledo, New classic MF ML54, Switzerland).

After five weeks of plant growth, the additional one hundred plants grown from water-soaked and bacterized seeds, were infested with 15 *D. radicum* neonates, five *P. xylostella* L2 larvae or five *M. brassicae* neonate larvae. Plants were first covered in nylon mesh bags. Larvae of *M. brassicae* and *P. xylostella* were placed on a leaf with a paint brush, whereas *D. radicum* larvae were brushed onto the carefully exposed main root. We measured *P. xylostella* and *M. brassicae* individual larval biomass after 10 days of feeding by weighing on a microbalance (CP2P, Sartorius AG, Germany) to the nearest 0.001 mg. Plant shoots were harvested after a total of six weeks of plant growth and weighed as described above. Adult *D. radicum* flies were caught in the mesh bags after emergence and collected once every 24 h, and weighed to the nearest 0.001 mg (CP2P, Sartorius AG, Germany). The remaining plant shoots were harvested after a total of 12 weeks of plant growth. This comprised experiment 1.

In the second experiment, we grew two sets of bacterized seeds and water-soaked seeds using the same method as described above, in similarly treated soil and in similar growth conditions. The plants intended for each insect infestation were grown six weeks apart due to limited greenhouse space. We infested the plants after five weeks of plant growth, in respective treatment with 20 *D. radicum* neonates or 10 *P. xylostella* L2 larvae. *Plutella xylostella* larvae were individually weighted after 10 days of feeding, and plant shoots were harvested, after a total of

six weeks of plant growth. Pupae of *D. radicum* were collected from the soil after 3 weeks of infestation, and placed in a Petri dish until emergence, adult flies being collected daily and then weighed. The plants were harvested after eight weeks of plant growth. The aboveground dry plant biomass was determined as described above.

Statistical analysis

Plant data were analyzed with GLM with a gamma distribution or ANOVA, where the model with lowest AIC score was used (according to REML criteria). Insect data were analyzed with GLMM with plant as a random factor. The statistical tests were performed using R version 3.5.0, with R Studio version 1.1.423 (R Core Team, 2018), specifically the packages car (Fox & Weisberg, 2019), lmtest (Zeileis & Hothorn, 2002), readxl, ggplot2, fitdistrplus and lme4 (Bates *et al.*, 2015).

Results

Shoot dry biomass

To investigate whether seed sterilization and seed bacterization interfered with germination and plant growth, we measured seed germination and plant growth for the four seed treatments studied in experiment 1. The proportion seed germination was 93-100% for the four seed treatments (Supplemental Fig. **S1**). Shoot dry biomass was similar for the four seed treatments after 6 weeks of plant growth (Fig. **1**, ANOVA, $F = 1.08$, df = 3, $p = 0.37$).

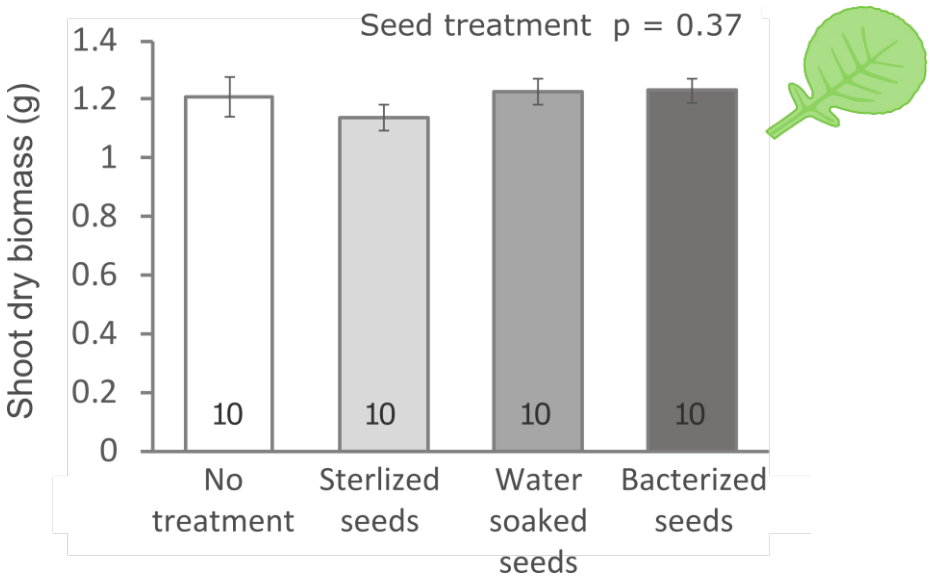

Figure 1 *Brassica oleracea* plant shoot dry biomass after six weeks of growth from four seed treatments. No treatment: control plants grown from untreated seeds. Sterilized seeds: plants grown from sterilized seeds. Water-soaked seeds: plants grown from sterilized seeds soaked in sterilized 10 mM $MgSO_4$. Bacterized seeds: plants grown from sterilized seeds soaked in rhizobacterial suspension (1×10^9 CFU ml^{-1}) for 4 h, and then dried for 24 h. The numbers in the bars represent the number of plants in each treatment, the p-value is based on ANOVA ($\alpha = 0.05$). Bars show mean ± SE.

Influence of *P. simiae* seed bacterization on shoot dry biomass

To further assess the effect of bacterization on plant growth and herbivore performance, we grew *B. oleracea* bacterized and water-soaked seeds, and after five weeks infested the plants with one of three herbivore species, in two temporally separated experiments. In experiment 1, seed bacterization did not affect plant shoot dry biomass (ANOVA, $F = 0.20$, df = 1, p = 0.65; Fig. **2A**). Infestation with *M. brassicae* and *P. xylostella* did not affect plant shoot dry biomass (ANOVA, $F = 0.82$, df = 2, p = 0.45; Fig. **2A**). Plants infested with *D. radicum* had a lower plant shoot dry biomass when harvested after 12 weeks (ANOVA, $F = 42.65$, df = 1, $p < 0.001$; Fig. **2B**), but seed bacterization showed no effect (ANOVA, $F = 1.14$, df = 1, p = 0.30; Fig. **2B**). Furthermore, in experiment 2 seed bacterization did not affect plant biomass (ANOVA, $F = 1.27$, df = 1, p = 0.27; Fig. **2C**) after 6 weeks of plant growth. *Plutella xylostella* infestation did not influence plant dry shoot biomass (ANOVA, $F = 0.32$, df = 1, p = 0.58, Fig. **2C**). Plant growth in the second seed set intended for *D. radicum* infestation was slow due to unknown reasons. Nevertheless, the results showed that *D. radicum* herbivory affected plant biomass (ANOVA, $F = 6.18$, df = 1, p = 0.015, Fig. **2D**), whereas plant

shoot biomass was not affected by seed bacterization after 8 weeks (ANOVA, $F = 1.32$, df = 1, $p = 0.26$, Fig. **2D**).

Influence of P. simiae seed bacterization on insect biomass

In our experiments herbivore performance, evaluated as insect biomass, was measured as a proxy for plant resistance. In experiment 1, seed bacterization did not affect *M. brassicae* and *P. xylostella* biomass after feeding on plants for 10 days, or *D. radicum* biomass at adult emergence from the soil (Fig. **3A**, *P. xylostella*: GLMM, $\chi^2 = 1.07$, df = 1, $p = 0.30$. Fig. **3B**, *M. brassicae*: GLMM, $\chi^2 = 2.49$, df = 1, $p = 0.12$. Fig. **3C**, *D. radicum*: GLMM, $\chi^2 = 0.13$, df = 1, $p = 0.72$). In experiment 2, *P. xylostella* larval biomass was not affected by seed bacterization after feeding for 10 days (Fig. **3D**: GLMM, $\chi^2 = 1.65$, df = 1, $p = 0.20$). *Delia radicum* adult weight was positively affected by seed bacterization after extraction as pupae from the soil, three weeks after the onset of infestation (Fig. **3E**: GLMM, $\chi^2 = 18.52$, df = 1, $p < 0.001$).

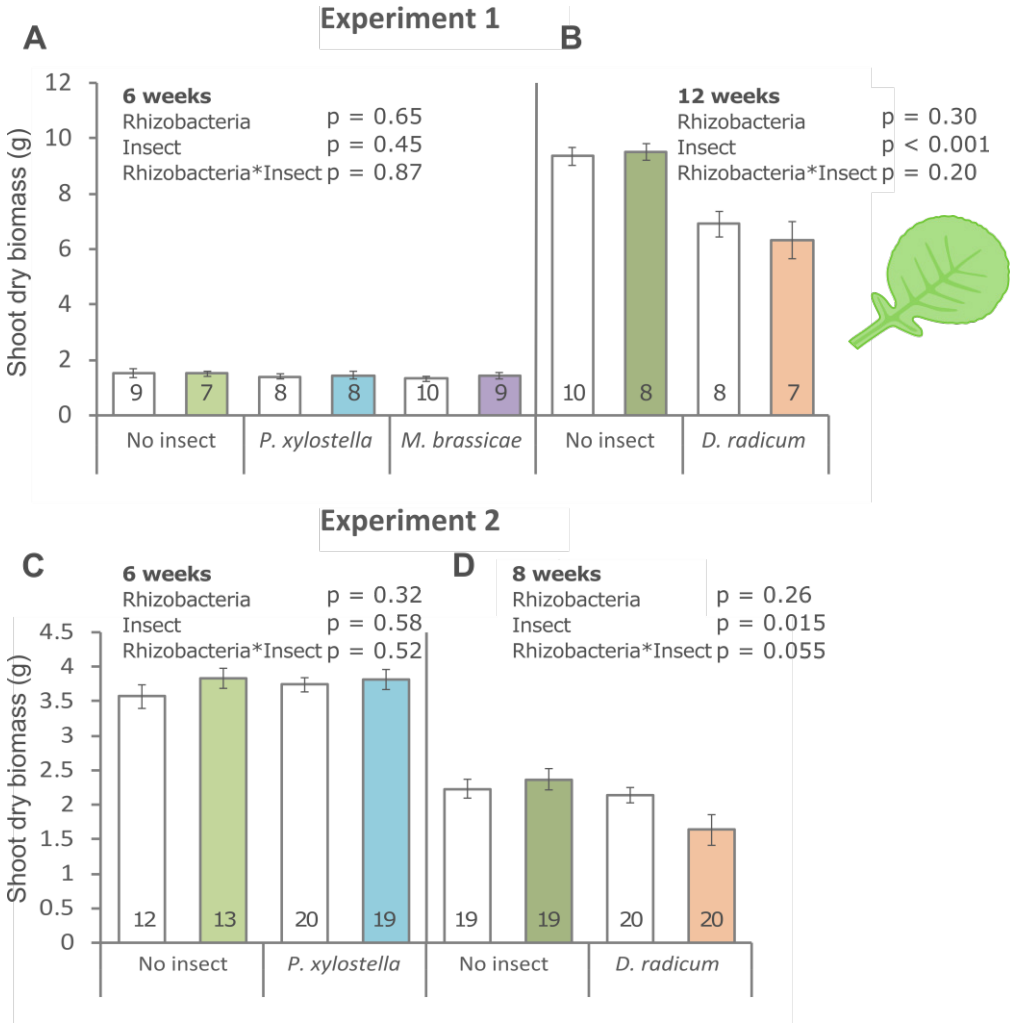

Figure 2 Shoot dry biomass of plants grown from water-soaked or bacterized seeds. **A.** Shoot dry biomass of plants grown from water-soaked seeds (white bars) or bacterized seeds (colored bars), uninfested (no insect) or infested with *Plutella xylostella* or *Mamestra brassicae* larvae; **B.** Shoot dry biomass of plants grown from water-soaked seeds (white bars) or bacterized seeds (colored bars), uninfested (no insect) or infested with *Delia radicum* after 12 weeks. **C.** Shoot dry biomass of plants grown from water-soaked seeds (white bars) or bacterized seeds (colored bars) and grown six weeks apart, infested with *P. xylostella*. **D.** Shoot plant dry biomass of plants grown from water-soaked seeds (white bars) or bacterized seeds (colored bars), uninfested (no insect) or infested with *D. radicum*, after eight weeks. Numbers in the bars represent the number of plant replicates, the p-values are based on ANOVA ($\alpha = 0.05$), and the bars show mean ± SE.

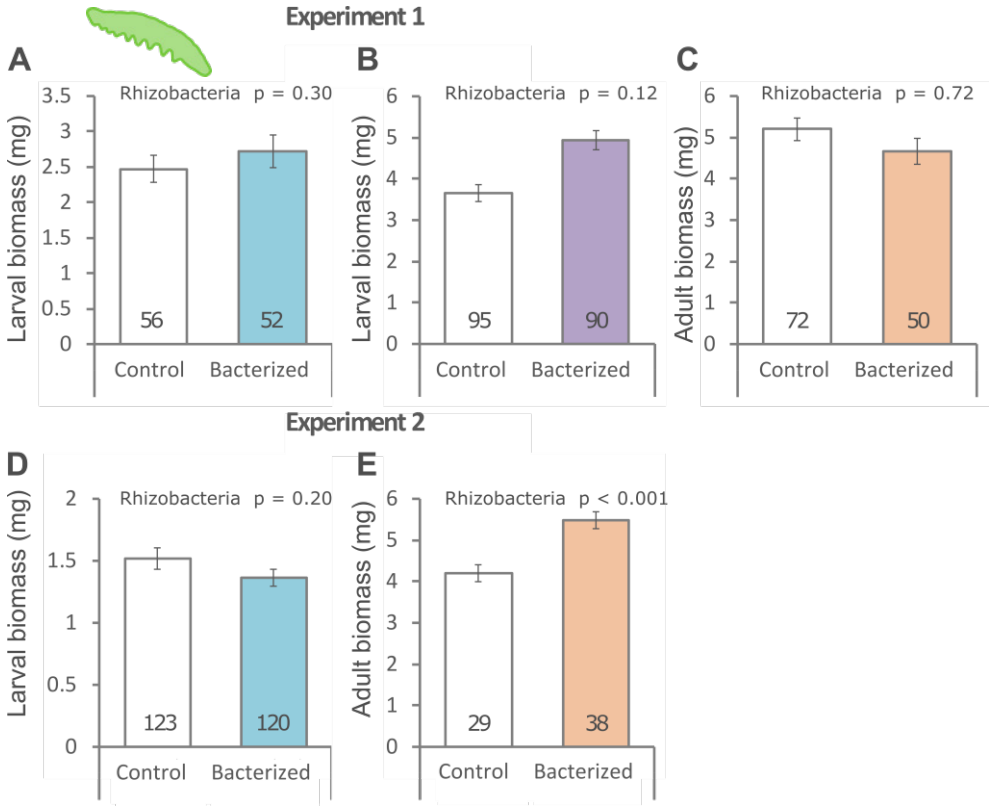

Figure 3 Insect biomass when feeding on plants grown from water-soaked or bacterized seeds. **Experiment 1. A.** *Plutella xylostella* larval biomass after feeding on control plants grown from water-soaked seeds (white bar) or on plants grown from bacterized seeds (colored bar) for 10 days. **B.** *Mamestra brassicae* larval biomass after feeding on control plants grown from water-soaked seeds (white bar) or on plants grown from bacterized seeds (colored bar) for 10 days. **C.** *Delia radicum* adult biomass after soil emergence; larvae had been feeding on control plants grown from water-soaked seeds (white bar) or on plants grown from bacterized seeds (colored bar). **Experiment 2. D.** *Mamestra brassicae* larval biomass after feeding on control plants grown from water-soaked seeds (white bar) or on plants grown from bacterized seeds (colored bar) for 10 days. **E.** *Delia radicum* adult biomass after soil emergence; larvae had been feeding on control plants grown from water-soaked seeds (white bar) or on plants grown from bacterized (colored bar). Pupae were collected from soil and kept until emergence and then weighed. Numbers in the bars represent the number of measured insects, the p-values are based on GLMM ($\alpha = 0.05$). Bars show mean ± SE.

Discussion

Several studies have examined the seed bio-priming method to enhance yield or biomass production (Raj *et al.*, 2004; Abuamsha *et al.*, 2011b; Manrique *et al.*, 2019), yet few studies have included plant resistance to insect herbivores (Crialesi *et al.*, 2017; Thuler *et al.*, 2017). Therefore, we investigated cabbage bacterization effects on plant biomass and resistance to three common insect herbivores of cabbage plants. The plants did not exhibit growth promotion as a result of bacterizing of the seeds. In two separate experiments, *D. radicum* infestation decreased plant biomass but there was no significant interaction with bacterization treatment. Insect performance was similar for insects feeding on plants grown from either bacterized or water-soaked seeds, but *D. radicum* biomass response to bacterization varied between our two experiments; in one we found no effect, in a second experiment *D. radicum* adult biomass was higher on plants grown from bacterized seeds.

Although the results suggest that seed bacterization with *P. simiae* WCS417r did not increase *B. oleracea* plant biomass, previous research showed that rhizobacterial growth promotion was recorded for cabbage plants upon the application of other delivery methods and bacterial strains. For example the strain *Bacillus megaterium* TV-91C increased cabbage growth by 22.6 % after soaking seeds in rhizobacterial solution (Turan *et al.*, 2014). Additionally, the strain *P. simiae* WCS417r has before been shown to promote cabbage growth when mixed into sterilized soil (Friman *et al*., 2021a). A possible explanation for the discrepancy between these results and those from earlier studies could be the lower amount of inoculum found on the seeds in this study. A higher amount of inoculum was measured on seeds in other studies (Szczech & Shoda, 2006; Abuamsha *et al.*, 2011b). However, an increasing amount of seed inoculum does not necessarily lead to plant growth promotion (Ciccillo *et al.*, 2002). The optimal amount of inoculum per seed for successful colonization upon application of seed biopriming is unknown.

It is important to notice that the specific bacterial delivery method can result in different effects on plant growth (Szczech & Shoda, 2006). Adding *Burkholderia ambifaria* MCI7 to the soil impaired maize plant shoot fresh weight but not root fresh weight, compared to non-inoculated plants. On the other hand, seed bio-priming improved plant root fresh weight but not shoot fresh weight (Ciccillo *et al.*, 2002). Thus, rhizobacterial delivery method may affect growth promotion and root-shoot ratio. It is unknown if root growth in our experiment was promoted, as we did not measure root biomass.

Since the type of rhizobacterial delivery method affects plant growth differently, it may be that the delivery method also affect insect

herbivore performance differently. Therefore, herbivore performance in this study may not be compared to results of herbivore performance in studies where rhizobacteria are mixed into the soil. Even though the results presented here are difficult to link to other plant-microbe-insects studies due to differences in inoculation methods, several promising studies hint at the capacity of beneficial microbes to enhance plant resistance against insects through seed bacterization (Fahimi *et al.*, 2014; Gadhave *et al.*, 2016a; Disi *et al.*, 2018). These studies were performed using a seed soak without drying, which may be less applicable in agriculture (Rocha *et al.*, 2019). More research is needed to unravel the mechanisms underlying these differences between inoculation methods, and to standardize the seed-biopriming method.

The results in the present study show that *P. simiae* seed bacterization did not have an effect on larval biomass of *P. xylostella* and *M. brassicae*. In a similar study where bio-priming was used, Thuler *et al.* (2017) showed that cabbage resistance against *P. xylostella* is improved by seed bio-priming with *Kluyvera ascorbata* EN4 and *Bacillus thuringiensis* HPF14 (Thuler *et al.*, 2017). The insects were feeding on leaf discs from leaves previously mechanically damaged, which is a difference in the experimental setup compared to the study presented here. These experimental setup differences may have caused the difference in results; setup differences included e.g. insect infestation methods, bacterial load (CFU/seed), seed drying after bacterization and implementation of mechanical damage on leaves.

The specificity related to plant and bacterial strain is an essential part of rhizobacterial inoculation (Drogue *et al.*, 2012; Chamam *et al.*, 2013). This strain specificity can also affect plant response towards insects. Among the strains *Pseudomonas fluorescens* UTPF68, UTPF1, UTPF6 and PF169, only inoculation with PF169 increased cucumber resistance against the cotton aphid *Aphis gossypii*, when seeds were bacterized (Fahimi *et al.*, 2014). We therefore speculate that not only the rhizobacterial delivery method, but also the specific bacterial strain used for bio-priming plays an important role in the bacterial colonization and effectiveness.

Effects on *D. radicum* biomass varied between experiments in this study: the first experiment exhibited no difference whereas the second experiment showed an increase of fly biomass when insects fed on plants grown from bacterized seeds. Despite the variation in the effect on *D. radicum* adult weight, previously an increase in weight has been found from rhizobacterial addition by mixing into soil (Friman *et al*., 2021a). Several factors may have influenced the insect biomass. The experiments in this study were temporally separated, allowing for seasonal changes

affecting the plants and fly colony. Furthermore, differences between soil batches may influence insect response to rhizobacterial soil inoculation (Pangesti *et al.*, 2015a).

Inoculating seeds can serve as a route of transportation and dissemination of the bacteria to the plant root. It has been hypothesized that an early introduction in the seedling phase may help to steer rhizobacterial colonization towards the strains that are most beneficial for yield (Rocha *et al.*, 2019). In support of this, studies show that microbiome assembly may be most dynamic in the first two weeks of plant life (Torres-Cortes *et al.*, 2018; Tkacz *et al.*, 2020). Seedlings likely assemble their microbial community from both surrounding soil and from the seed microbiome (Bulgarelli *et al.*, 2013; Nelson, 2018). However, the question of an optimal point in time in the plant life cycle to nudge microbiome community with the addition of beneficial rhizobacteria is still uncharted.

This study demonstrates that *B. oleracea* seeds bacterized with the strain *P. simiae* WCS417r according to the bio-priming method, did not promote plant shoot biomass or enhanced plant resistance. Our results even suggest that the performance of the belowground insect herbivore *D. radicum,* was increased as a result of bacterial treatment. The selection of both the strain and the delivery method may be essential for optimizing rhizobacterial inoculations in agriculture. Our results underline the complexity of rhizobacteria-plant interactions, and further research should investigate the factors influencing the effects of bacterization on plant growth and defense.

Acknowledgments

The authors greatly appreciate the support by the insect rearing staff of the Laboratory of Entomology for providing the *Plutella xylostella* and *Mamestra brassicae* used in this study. The authors would like to especially thank the *Delia radicum* rearing team: Peter Karssemeijer, Kay Moisan and Max Wantulla. This work was funded by Wageningen University and private funding.

Author contributions

JF, RP and TW conducted the experiments. JF wrote the manuscript with the support of MD and JJAvL. All authors read and approved the manuscript.

Supplemental information

Table S1 Seed germination per day observed from seed treatments. No treatment: plants grown from untreated seeds. Sterilized: plants grown from sterilized seeds. Water-soaked seeds: plants grown from seeds soaked in sterilized 10 mM $MgSO_4$ for 4 h. Bacterized seeds: plants grown from seeds soaked in rhizobacterial suspension in 10 mM $MgSO_4$ (1×10^9 CFU ml^{-1}) for 4 h.

		Number of seeds			
	Day	No treatment	Sterilized seeds	Water-soaked seeds	Bacterized seeds
Total	1	95	16	15	15
Germination/day	2	4	6	8	3
Accumulative Germination/day	3	64	15	14	11
Accumulative Germination/day	4	88	15	14	15
Accumulative Germination/day	5	88	15	14	15
Percentage		93	94	93	100

Chapter 5

Rhizobacterial inoculation enhances plant growth and reduces leaf feeding during dual infestation with a leaf and a root herbivore

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Abstract

Plant growth-promoting rhizobacteria can modulate plant resistance against single insect herbivores. Yet, plants are often attacked by more than one insect herbivore. Here, we investigated rhizobacterial modulation of plant resistance under single and multiple herbivore attack. We grew cabbage plants, *Brassica oleracea*, in soil inoculated with the rhizobacterium *Pseudomonas simiae* WCS417r in a greenhouse, and infested them with caterpillars of the leaf-feeding diamondback moth, *Plutella xylostella*, and/or larvae of the root-feeding cabbage root fly, *Delia radicum*.

We found that rhizobacterial inoculation increased plant shoot dry mass. Furthermore, rhizobacterial inoculation lowered the area of leaf tissue consumed by *P. xylostella*, but not larval biomass. Root damage by *D. radicum* feeding was similar on plants grown in rhizobacterially inoculated and control soil. Rhizobacterial treatment did not affect gene expression levels of marker genes in the jasmonic acid pathway, whereas local insect feeding upregulated jasmonic-acid-associated gene expression. Lastly, rhizobacterial inoculation did not differently affect plant resistance against single infestation by *D*. *radicum* or *P*. *xylostella*, compared to double herbivory by both *D*. *radicum* and *P*. *xylostella*.

In conclusion, rhizobacterial soil inoculation promoted *B. oleracea* plant growth. We suspect there was no active rhizobacterial modulation of plant resistance, and therefore no different modulation of plant resistance in response to dual herbivory compared to single herbivory. These results highlight the difference between rhizobacterial plant growth promotion and rhizobacterial plant resistance modulation. Further, multiple herbivores on the same host plant may have a stronger effect on each other than rhizobacterial modulation of plant resistance.

Keywords Rhizobacterium *Pseudomonas simiae* WCS417r*,* diamondback moth *Plutella xylostella,* cabbage root fly *Delia radicum*, plant-insect interaction

Introduction

Plants are attacked by numerous insects both above- and belowground (Stam *et al.*, 2014). Herbivore attack may cause changes in the root microbiome (Dawson *et al.*, 2004; Dematheis *et al.*, 2012; Hu *et al.*, 2018; Ourry *et al.*, 2018), which in turn can affect plant-feeding insects (Berendsen *et al.*, 2012; Badri *et al.*, 2013; Hu *et al.*, 2018). Shoot- and root-feeding insect herbivores can affect each other *via* plant-mediated signaling (Erb *et al.*, 2009; Papadopoulou & van Dam, 2017; Karssemeijer *et al.*, 2020). Still, it is unclear whether beneficial microbes modify plant resistance against simultaneously feeding above- and belowground insects.

Plant resistance against herbivores is regulated by phytohormonal signaling pathways both above- and belowground (Bezemer & van Dam, 2005; Erb *et al.*, 2012b; Mithofer & Boland, 2012; Verma *et al.*, 2016). A major phytohormonal signaling pathway influencing chewing insect herbivores is the jasmonic acid/ethylene pathway. Jasmonic acid, or jasmonic acid-derived signals, are transported *via* vascular bundles throughout the plant (Ruan *et al.*, 2019), and induce phenotypic changes systemically (Kaplan *et al.*, 2008). As a result of systemic responses to herbivory, herbivores can influence each other through plant-mediated alterations of defense and nutrient allocation throughout the plant (Johnson *et al.*, 2013; Huang, W *et al.*, 2014).

Beneficial rhizobacteria may induce resistance to insect herbivores (Pineda *et al.*, 2010; Friman *et al.*, 2021a) and a functional jasmonic acid signaling pathway is required for this (Pieterse *et al.*, 2012; Pieterse *et al.*, 2014; Rasmann *et al.*, 2017). However, experiments with plants colonized by rhizobacteria show that their effects on a single insect can vary from positive to negative (Megali *et al.*, 2014; Santos *et al.*, 2014; Megali *et al.*, 2015; Pangesti *et al.*, 2015a). Nevertheless, plants are not interacting with single herbivores one at a time, but are often attacked by several insects simultaneously (Stam *et al.*, 2014). It is yet unknown how rhizobacteria modulate plant-mediated interactions between two herbivore species feeding on the plant at the same time.

Among crop plants, the *Brassica* genus is one of the most commonly cultivated, of which cabbage (*Brassica oleracea* L.) is prominent and grown worldwide. Cabbage plants are damaged by several insect pest species. A common and highly destructive insect pest is the diamondback moth *Plutella xylostella* L. (Zalucki *et al.*, 2012; Furlong *et al.*, 2013). The females deposit eggs singly or in small groups, and the first instar larvae mine into the leaves. *Plutella xylostella* exhibits multiple resistance to insecticides (Furlong *et al.*, 2013), prompting development of other, sustainable, control measures. The most important belowground

insect pest of cabbage is the cabbage root fly *Delia radicum* L. (EIP-AGRI, 2016). The female flies deposit their eggs near the stem base of the plant. The hatched larvae mine into the root, and later pupate in the soil. Root miners such as *D. radicum* are difficult to effectively control with traditional methods in agriculture.

Here, we asked whether plant growth-promoting rhizobacterium *Pseudomonas simiae* WSC417r modulates *B. oleracea* defense differently under dual herbivore attack compared to bacterial modification of plant defense under single herbivore attack. We quantified plant shoot dry biomass, measured consumed leaf area by *P. xylostella* and scored root damage by *D. radicum* under single and dual attack, on plants grown on soil with and without rhizobacterial soil addition. We compared the effects of single and dual attack by *P. xylostella* and *D. radicum* on the gene expression of marker genes in the JA signaling pathway in leaves and root of plants grown in inoculated or control soil.

Materials and methods

Insect rearing

Delia radicum L. (Diptera: Anthomyiidae), and *Plutella xylostella* L. (Lepidoptera: Plutellidae) were reared at 22 ± 2°C, 40-50 % RH, 16:8 h (light:dark). Adult flies were fed on a 1:1:1 mix of milk powder, sugar and yeast flakes, in the presence of honey. Larvae were reared on roots of rutabaga (*Brassica napus* L.). Flies oviposited on a fresh piece of rutabaga placed in a Petri dish on moist filter paper. The rutabaga piece was removed and the Petri dish was closed until hatching. *Plutella xylostella* was reared on *B. oleracea* L. *gemmifera* cv. Cyrus plants at 22 ± 2°C, 40-50 % RH, 16:8 h (light:dark). Second instar larvae (L2) were used in the experiments.

Rhizobacterium Pseudomonas simiae *WCS417r and plant growing conditions*

The rhizobacterium *Pseudomonas simiae* WCS417r, formerly *P. fluorescens* WSC417r (Berendsen *et al.*, 2015), was grown on King's B (KB) medium containing 25 µg ml^{-1} rifampicin at 28°C for 48 h. This strain is a plant growth promoting rhizobacterium, originally isolated from wheat rhizosphere. A bacterial solution was made by harvesting bacteria from the agar plates and suspending them in sterilized 10 mM $MgSO_4$. The solution was adjusted to a cell density of 1×10^9 colony forming units (CFU) per ml (OD_{660} = 1.0).

White cabbage seeds (*Brassica oleracea* cv. Christmas Drumhead, Centre for Genetic Resources, Wageningen, The Netherlands) were surface-sterilized by submerging the seeds in 80 % ethanol for 1 min,

followed by 15 min in 1 % hypochlorite. The seeds were then washed three times with sterilized tap water.

Potting soil (Horticoop b.v., Slingerland Potgrond) was mixed 1:3 with Perlite (Agra-perlite, grain 3) and autoclaved twice (121°C, 20 minutes, 24 h in between sterilizations). The bacterial solution was mixed into the soil at 50 ml kg^{-1}. For control soil treatment, 10 mM $MgSO_4$ sterilized solution was added at the same dose. The sterilized seeds were added to the soil in a sterilized seedling tray. After one week of plant growth, the seedlings were transplanted into clean 11 x 11 x 12 cm pots. In total 240 plants were grown, of which 120 plants were used for assessing insect and plant performance, and 120 plants for sampling tissue that was later used for gene expression analysis. Plants were watered when needed, around three times per week until the soil was moist, and Hyponex® fertilizer 50 ml was added once per week after transplant. Plants were grown at 20 ± 2°C and 70 ± 10 % RH in a greenhouse. Photoperiod was maintained at 16:8 h (light:dark). When photosynthetic active radiation (PAR) dropped below 400 µmol $s^{-1}m^{-1}$, additional lighting was provided by halide bulbs (400 W). The plants used in the experiments were four weeks old.

Insect performance, plant growth and plant tissue sampling

Plants were randomly divided into five blocks, and assigned to a treatment. All plants were covered by a nylon mesh bag with an elastic band around the pot, allowing insects to move freely on the plant they were confined on. Plants received either five *P. xylostella* L2 larvae placed on the top leaves, or ten neonate *D. radicum* larvae that were placed next to the exposed stem base.

After 24 h of insect infestation, leaf samples were taken with a 1 cm diameter steel cork borer and immediately frozen in liquid nitrogen. For aboveground insects, the leaf disk was collected close to *P. xylostella* damaged leaf tissue. For *D. radicum* infested and non-infested plants, leaf samples were taken from the first fully expanded leaf from the top. Roots were collected, quickly washed, and frozen in liquid nitrogen. Plant tissues were stored at -80°C until analysis.

Plutella xylostella caterpillars were collected after 10 days of feeding and weighed on a microbalance to the nearest 0.001 mg (CP2P, Sartorius AG, Germany). Leaf feeding damage was quantified by measuring the leaf area consumed. Leaves were cut from the stem and taped carefully to a blank paper together with 1 cm^2 of paper as calibration area, and scanned (RICOH MPC4503). In Adobe Photoshop (v 19.1.7) scans were contrasted and pixels of missing leaf area were counted relative to the number of pixels of the calibration area to quantify the consumed leaf area. Each leaf was measured twice, and the average of

the two measurements was used. The consumed area was then divided by the number of caterpillars recorded for that plant. Root damage was scored by visual estimation after two weeks of *D. radicum* infestation. The root-damage scale of Wang *et al.* (2016) was used. In short, a score was given between 0 and 6, where 0 = no root damage and 6 = severely affected root tissue and only a small core of the tap root is left. A score of 1 represents feeding comprising less than 10 % of the root surface area; a score of 2 represents 11–25 % root damage of the surface area; a score of 3 represents 26–50 % damage; a score of 4 represents 51–75 % and a score of 5 represents 76–100 % damage; lastly a score of 6 = a root is completely eaten or almost completely eaten.

After *P. xylostella* collection or *D. radicum* damage scoring, plant shoots were harvested, dried at 70°C for 48 h, and weighed to the nearest 0.1 mg (Mettler Toledo, New classic MF ML54, Switzerland).

Plant gene expression analysis

We quantified gene expression in leaves for: (1) the JA associated *LIPOXYGENASE-2* (*LOX2*), (2) transcription factor *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* (*ORA59*), a main integrator of the JA- and ET-signaling pathways and (3) transcription factor *MYC2/JASMONATE INSENSITIVE1* (*MYC2*), a main integrator of the JA- and ABA-signaling pathways. For roots, we quantified gene expression of (1) *LIPOXYGENASE-6* (*LOX6*), (2) *ORA59,* (3) *MYC2*, and (4) *ALLENE-OXIDE SYNTHASE* (*AOS*), a gene in the JA biosynthetic pathway. The primers used are presented in Supplemental Table **S1**. Tissue was ground with a pestle in an Eppendorf tube whilst cooled with liquid nitrogen. RNA was isolated with an RNA extraction kit according to the manufacturer's protocol (Isolate II RNA Plant Kit, Bioline). Synthesis of cDNA was carried out with a cDNA synthesis kit (SensiFAST, Bioline) following the manufacturer's instructions and diluted five times. To control for RNA quantity, the housekeeping genes *SAR1a*, *PER4* and *Btub* were used for leaf tissue, whereas *Act-2* and *Btub* were used for root tissue. Efficiency and optimal annealing temperature of each primer was determined before qRT-PCR analysis (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). Reaction mixtures (20 µl) per sample contained 5 µl of five times diluted cDNA, 0.4 µl forward primer 25 mM, 0.4 µl reverse primer 25 mM, 10 µl qPCR SYBR green mix (Bio-rad), and 4.2 µl water. The thermocycle parameters for leaf tissue, initial polymerase activation during 10 min at 95°C, 40 cycles of 30 s at 95°C, 60 s at 57°C and 30 s at 72°C. For root tissue another sequence of temperatures was used: 10 min at 95°C, 40 cycles of 30 s at 95°C, 60 s at 62°C and 30 s at 72°C.

Statistical analysis

Insect and plant performance data were analyzed with a Generalized Linear Mixed Model (GLMM), built from the model including all factors, with the lowest AIC score (according to REML criteria). For insect-related data, plant and block were used as random factors. For plant data, block was used as random factor. Significance level was set to $\alpha = 0.05$. When relevant, post-hoc Tukey tests were run with a maximum likelihood fit (Laplace Approximation). The statistical tests were carried out with R version 3.5.0 and R Studio version 1.1.423 (R Core Team, 2018) using packages car (Fox & Weisberg, 2019), lmtest (Zeileis & Hothorn, 2002), readxl, ggplot2, fitdistrplus and lme4 (Bates *et al.*, 2015).

Results

Effect of rhizobacterial inoculation and double infestation on P. xylostella performance

To explore whether rhizobacterial inoculation and concurrent *D. radicum* infestation affected the amount of *P. xylostella* feeding, we quantified the leaf area consumed. Plant rhizobacterial inoculation resulted in a smaller amount of leaf area consumed by *P. xylostella* (Fig. **1**; GLMM: $\chi^2 = 11.13$, df = 1, $p < 0.001$). Simultaneous feeding by *D. radicum* did not influence the leaf area consumed by *P. xylostella* (GLMM: $\chi^2 = 1.58$, df = 1, $p = 0.21$). The interaction between the main factors insect treatment and rhizobacterial inoculation was not significant (GLMM: $\chi^2 = 0.10$, df = 1, $p = 0.75$), indicating that rhizobacterial inoculation affected *P. xylostella* feeding similarly in the presence or absence of *D. radicum*.

To further assess the differences in *B. oleracea* resistance to *P. xylostella* after rhizobacterial inoculation and *D. radicum* infestation, we measured *P. xylostella* larval biomass. We found that plant rhizobacterial inoculation did not affect *P. xylostella* larval biomass (Fig. **2**; GLMM: $\chi^2 = 0.29$, df = 1, $p = 0.59$). In contrast, dual infestation by both *D. radicum* and *P. xylostella* resulted in lower *P. xylostella* biomass (GLMM: $\chi^2 = 27.19$, df = 1, $p < 0.001$). The interaction between rhizobacterial inoculation and insect treatment was not significant (GLMM: $\chi^2 = 1.97$, df = 1, $p = 0.16$), indicating that rhizobacterial inoculation affected *P. xylostella* biomass similarly in the presence or absence of *D. radicum*.

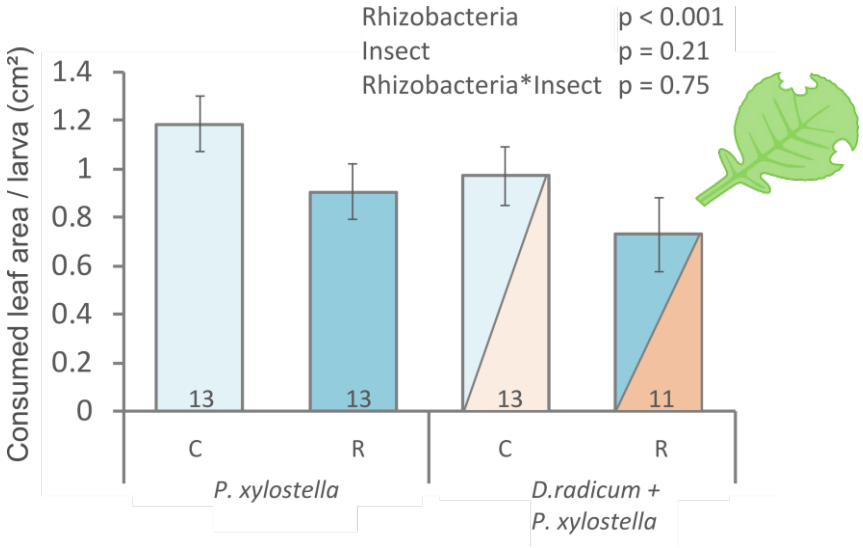

Figure 1 Leaf area consumed by *Plutella xylostella* when feeding on *Pseudomonas simiae* inoculated (R) or control (C) plants as the only herbivore, and when co-feeding with *Delia radicum*. Consumed leaf area was measured after 10 days of herbivore feeding. Double colored bars represent dual infestation with *P. xylostella* and *D. radicum*. The p-values are based on GLMM analysis (α = 0.05). Numbers in the bars represent the number of plants; bars show mean ± SE.

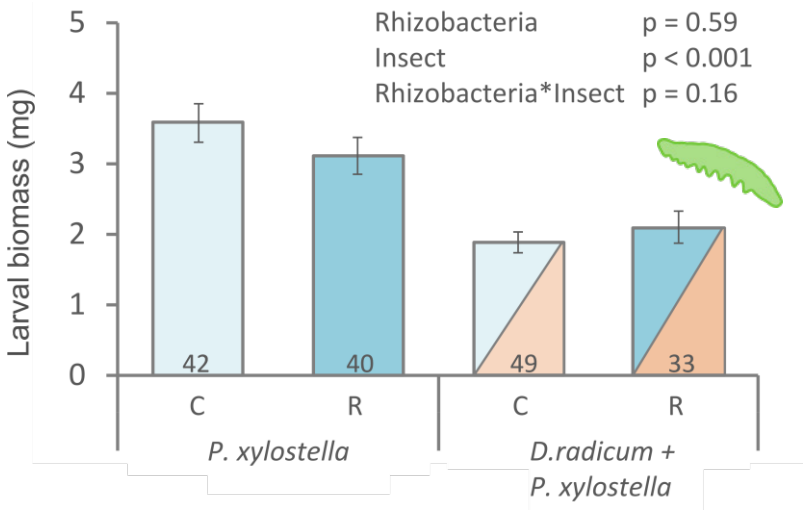

Figure 2 *Plutella xylostella* larval biomass after feeding on *Pseudomonas simiae* inoculated (R) or control (C) plants, and co-feeding with *Delia radicum* or feeding as only herbivore on the plant. Biomass was measured after 10 days of herbivore feeding. Double colored bars represent simultaneous feeding by the two herbivores *P. xylostella* and *D. radicum*. The p-values are from GLMM analysis (α = 0.05). Numbers in the bars represent the number of caterpillars; bars show mean ± SE.

Effect of rhizobacterial inoculation and double infestation on plant performance

To measure herbivore damage belowground, we visually scored the extent of damage on primary roots on a scale of 1 to 6, where 1 represents the lowest and 6 the largest extent of root damage. We found that rhizobacterial inoculation did not influence *D. radicum* root damage (Fig. **3**; GLMM: $\chi^2 = 0.78$, df = 1, p = 0.38). Likewise, additional infestation by *P. xylostella* did not affect the amount of *D. radicum* root damage compared to root damage when *D. radicum* was the only herbivore (GLMM: $\chi^2 = 0.84$, df = 1, p = 0.36). The interaction between the bacterial inoculation and type of insect herbivore was not significant (GLMM: $\chi^2 = 0.04$, df = 1, p = 0.85), indicating that rhizobacterial inoculation affected *D. radicum* damage similarly in the presence or absence of *P. xylostella*.

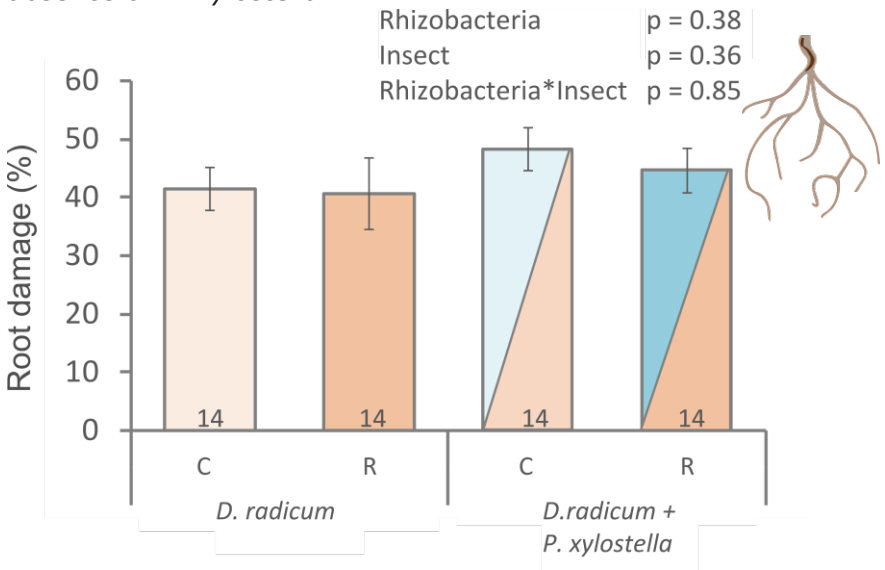

Figure 3 *Delia radicum* root damage in the presence (R) or absence (C) of *Pseudomonas simiae* inoculated in the soil, and on 6-week-old *Brassica oleracea* plants in the presence or absence of *P. xylostella* caterpillars feeding on the leaves. Root damage was visually measured as percentage discolored surface area. Double colored bars represent dual infestation by *D. radicum* and *P. xylostella*. The p-values are from GLMM analysis (α = 0.05). Numbers in the bars represent the number of plants; bars show mean ± SE.

To determine the effect of rhizobacterial inoculation on the growth of plants, we measured shoot dry biomass of plants with root herbivory, shoot herbivory or dual infestation. Shoot biomass of the plants was differentially affected by the treatments. The main factors rhizobacterial inoculation and insect treatment affected plant shoot dry biomass (Fig. **4**; GLMM; rhizobacterial inoculation: $\chi^2 = 22.79$, df = 1, p < 0.001; insect:

χ^2 = 21.43, df = 3, p < 0.001). Rhizobacterial inoculation resulted in larger shoot biomass. There was no significant interaction between type of insect herbivore and rhizobacterial inoculation (GLMM: χ^2 = 3.54, df = 3, p = 0.32), indicating that rhizobacterial inoculation affected plant growth similarly for all four herbivore treatments.

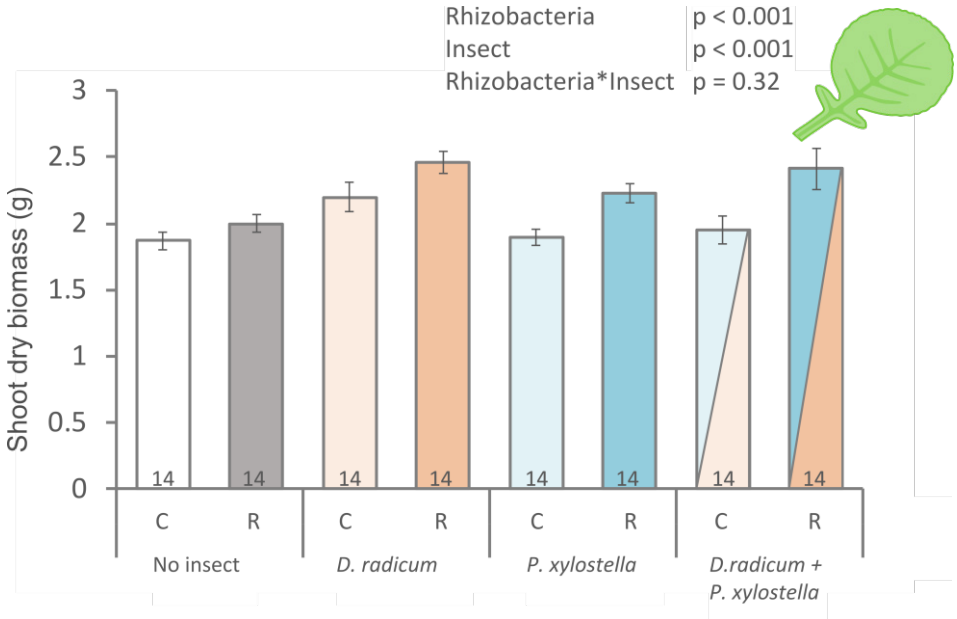

Figure 4 *Brassica oleracea* shoot dry biomass after 6 weeks of growth in *Pseudomonas simiae* inoculated or control soil for four herbivore infestation treatments**.** Shoot plant dry biomass of control (C) or *P. simiae* inoculated plants (R) plants, without insect infestation (no insect), or infested with *Plutella xylostella*, infested with *Delia radicum*, or co-infested with *Plutella xylostella* and *Delia radicum*; double colored bars represent dual insect infestation. The p-values are from GLMM analysis (α = 0.05). Numbers in bars represent number of plants; bars show mean ± SE.

Effect of rhizobacterial inoculation on plant defense gene expression levels

Plant gene expression levels were analyzed for selected JA-associated marker genes after 24 h of insect infestation. In leaves, *LOX2* expression was significantly affected by insect species (Fig. **5A**; GLMM; χ^2 = 259.98, df = 3, p < 0.001) but not by rhizobacterial inoculation (Fig. **5A**; GLMM; χ^2 =0.15, df = 1, p = 0.70). The interaction between rhizobacterial inoculation and insect treatment was not significant (Fig. **5A**; GLMM; χ^2 = 3.16, df = 3, p = 0.37). Insect infestations that included *P. xylostella* had the highest expression levels but also root feeding by *D. radicum* upregulated *LOX2* expression in the leaves.

Expression levels of *MYC2* in the leaves were not influenced by insect infestation or rhizobacterial inoculation (Fig. **5B**; GLMM; *MYC2*: rhizobacterial inoculation: $\chi^2 = 0.26$, df = 1, p = 0.61; insect: $\chi^2 = 5.93$, df = 3, p = 0.12; rhizobacterial inoculation × insect: $\chi^2 = 0.42$, df = 3, p = 0.94). *ORA59* expression in the leaves was affected by insect infestation (Fig. **5C**; GLMM; $\chi^2 = 15.73$, df = 3, p = 0.001) but not by rhizobacterial inoculation (Fig. **5C**; GLMM; *ORA59*: $\chi^2 = 0.99$, df = 1, p = 0.32). *ORA59* expression was higher in plants infested with the root-feeding *D. radicum* than in plants infested by both the root feeder and the leaf feeder. The interaction between insect treatment and rhizobacterial inoculation was significant (Fig. **5C**; GLMM; $\chi^2 = 29.09$, df = 3, $p < 0.001$), indicating that rhizobacterial treatment affected *ORA59* expression differentially for the four insect treatments.

In the roots, transcript levels of the marker gene *LOX6* were influenced by insect feeding, but not by rhizobacterial inoculation (Fig. **6A**; GLMM; *LOX6*: rhizobacterial inoculation: $\chi^2 = 1.22$, df = 1, p = 0.27; insect: $\chi^2 = 13.34$, df = 3, p = 0.004). *LOX6* transcript levels in response to infestation by *D. radicum* plus *P. xylostella* were higher than in plants infested by *P. xylostella* only. The interaction between insect treatment and rhizobacterial inoculation was not significant (Fig. **6A**; GLMM; $\chi^2 = 0.49$, df = 3, p = 0.92), indicating that rhizobacterial inoculation affected *LOX6* expression similarly for the four insect treatments.

For *MYC2* expression in the roots, neither insect treatment nor rhizobacterial inoculation had a significant effect (Fig. **6B**; GLMM; *MYC2*: rhizobacterial inoculation $\chi^2 = 0.05$, df = 1, p = 0.83; insect: $\chi^2 = 3.78$, df = 3, p = 0.29; rhizobacterial inoculation × insect: $\chi^2 = 3.78$, df = 3, p = 0.014). Insect treatment significantly affected the overall expression of *ORA59* and *AOS* in roots whereas rhizobacterial treatment did not (Fig. **6C**; GLMM; *ORA59*: rhizobacterial inoculation: $\chi^2 = 0.02$, df = 1, p = 0.88; insect: $\chi^2 = 19.90$, df = 3, $p < 0.001$; rhizobacterial inoculation × insect: $\chi^2 = 0.50$, df = 3, p = 0.92) (Fig. **6D**; GLMM; *AOS*: rhizobacterial inoculation: $\chi^2 = 0.18$, df = 1, p = 0.67; insect: $\chi^2 = 11.07$, df = 3, p = 0.011; rhizobacterial inoculation × insect: $\chi^2 = 6.07$, df = 3, p = 0.11). *Delia radicum* treatment upregulated the expression of these genes, whereas *P. xylostella* did not, neither as single herbivore nor in combination with *D. radicum*.

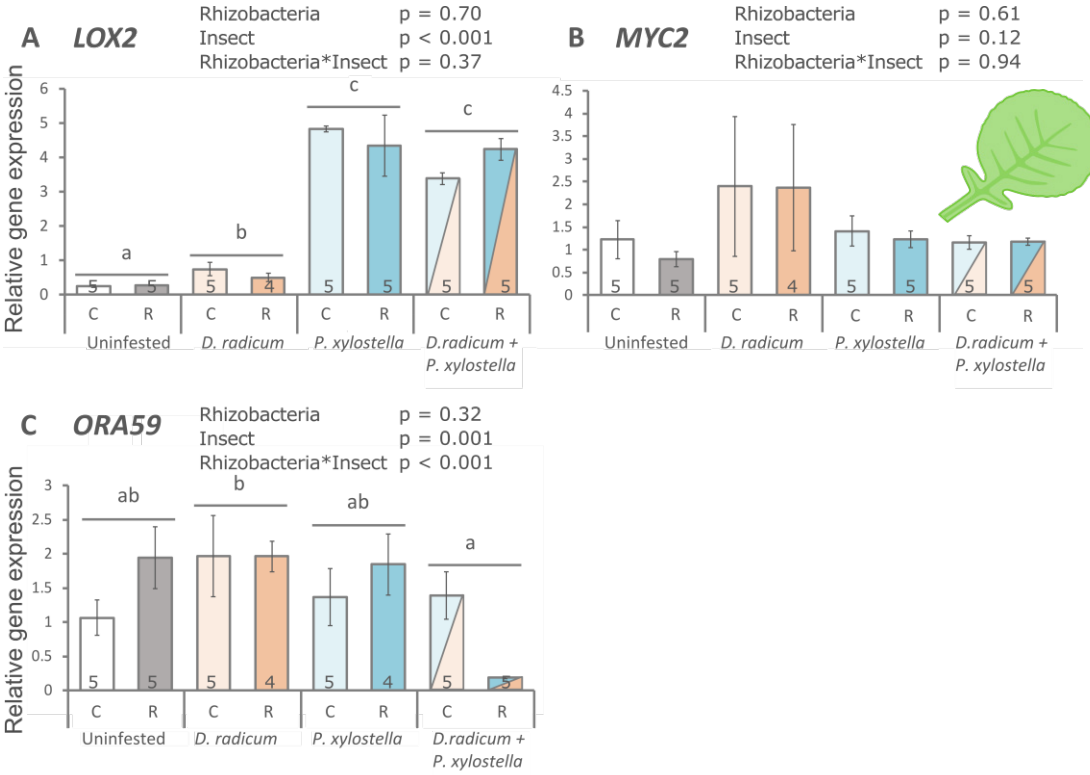

Figure 5 Expression levels of three marker genes in the jasmonic acid defense signaling pathway in leaves of *Brassica oleracea* plants grown in *Pseudomonas simiae* inoculated (R) or control (C) soil, 24 h after infestation with the root herbivore *Delia radicum*, the shoot herbivore *Plutella xylostella* or co-infestation with both herbivores, relative to the reference genes *SAR1a*, *PER4* and *Btub*. **A.** Relative expression levels of *LOX2*. **B.** Relative expression levels of *MYC2*. **C.** Relative expression levels of *ORA59*. Double colored bars represent dual infestation. Different letters indicate significantly different mean values between treatments, the p-values are from GLMM analysis (α = 0.05). Numbers in the bars represent the number of biological replicates; bars show mean ± SE.

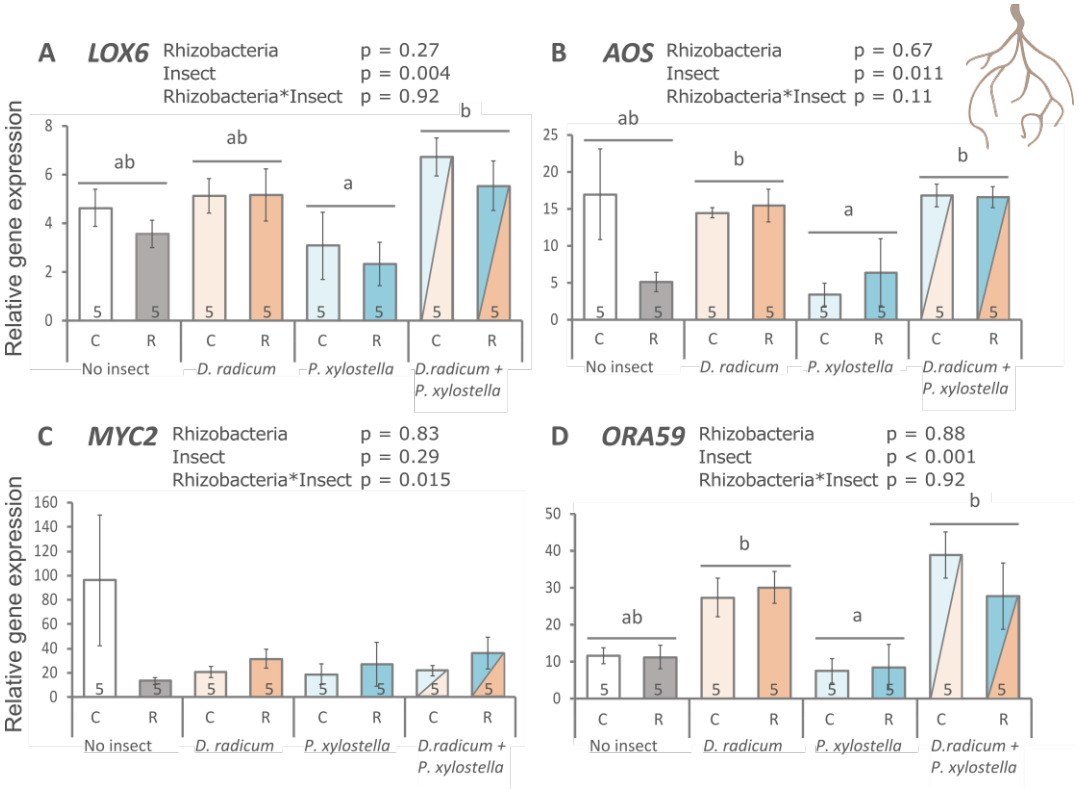

Figure 6 Expression levels of four marker genes in the jasmonic acid defense signaling pathway in primary roots of *Brassica oleracea* plants grown in *Pseudomonas simiae* inoculated (R) or control (C) soil, 24 h after infestation with the root herbivore *Delia radicum*, the shoot herbivore *Plutella xylostella*, both herbivores, or no insect, relative to the reference genes *Act-2* and *Btub*. **A.** Relative expression levels of *LOX6*. **B.** Relative expression levels of *MYC2*. **C.** Relative expression levels of *ORA59*. **D.** Relative expression levels of *AOS*. Double colored bars represent dual infestation. Different letters indicate significantly different mean values between treatments, the p-values are from GLMM analysis (α = 0.05). Numbers in bar represent the number of biological replicates; bars show mean ± SE.

Discussion

Plant-growth promoting microbes have received increasing attention from the agricultural sector as well as the scientific community. These microbes have been shown to modulate plant resistance against one insect herbivore species, yet plants are often hosts to more than one herbivorous insect species. Our study investigated the effects of rhizobacterial inoculation on dual infestation with a leaf- and a root-chewing herbivore. Although we found a plant growth promotion amongst plants grown in inoculated soil compared to plant grown in control soil, we did not find a decrease in insect weight due to rhizobacterial soil inoculation. However, we did measure a decrease in leaf area consumption by *P. xylostella* when feeding on plants grown in

rhizobacterially inoculated soil. We found no differences in *D. radicum* root damage between plants grown in inoculated soil compared to plants grown in control soil. Rhizobacterial effects on insect performance *via* plant mediated effects did not depend on type of herbivorous insect, *D. radicum* or *P. xylostella*, or simultaneous feeding from the above and belowground insects.

Although rhizobacterial soil inoculation increased plant shoot dry biomass of the plants grown in rhizobacterially inoculated soil compared to plant shoot dry biomass of plants grown in control soil, the inoculation did not affect larval weight or root damage. It is likely that that the plants growing in inoculated soil did not have an active ISR against the attacking insects. In addition, JA signaling was induced locally after insect feeding, but was not affected by rhizobacterial inoculation. The results showed no difference in JA signaling pathway regulation due to rhizobacterial inoculation, and therefore there was no further difference in JA signaling between a single and a dual infestation. These results are interesting, as it has earlier been established that plant growth promotion and ISR are two separately regulated phenomena in *A. thaliana* colonized by *P. simiae* WCS417r (Zamioudis *et al.*, 2013). Our results suggests a similar separation in *B. oleracea*, and further underline this distinction between rhizobacterial promoted plant growth and rhizobacterial increased plant resistance.

Rhizobacterial inoculation influenced the amount of insect shoot damage, but not root damage, independent of the presence of *D. radicum* larvae feeding on the roots. Rhizobacterial inoculation did not influence the plant resistance against double herbivory from both *D. radicum* and *P. xylostella*, but systemically reduced aboveground leaf herbivory by *P. xylostella*. We explain these results with increased plant nutritional value, which could lead to more efficient insect gut food conversion, when the insects fed on the plants grown from rhizobacterial inoculated soil. *Delia radicum* root damage may have been measured too early in the insect's development to yet show a difference between plants grown in inoculated soil and control plants.

Interestingly, a few other bacteria-plant-insect systems show a systemic negative effect from rhizobacterial inoculation on leaf area consumed by insects (Zehnder *et al.*, 1997; Commare *et al.*, 2002; Saravanakumar *et al.*, 2008). For example, leaf area consumed by *P. xylostella* was negatively affected, after *B. oleracea* seed inoculation with endophytic *Kluyvera ascorbata* EN4, when the insects fed on detached leaves (Crialesi *et al.*, 2017). On the other hand, neutral effects from microbes have previously also been recorded, where consumed leaf area by *Spodoptera littoralis* was not affected by presence of soil microbes in

the soil of maize plants (D'Alessandro *et al.*, 2014). However, the bacterial application methods and experimental setup differ between studies, which makes it problematic to compare results.

Root feeding insect can affect shoot feeders, and *vice versa* (Poelman *et al.*, 2008; Kafle *et al.*, 2017). In the present study, the double infestation by *D. radicum* and *P. xylostella* resulted in lower *P. xylostella* biomass, regardless of rhizobacterial inoculation. The lower larval biomass could be explained by three mechanisms. Root damage by *D. radicum* could have negatively influenced plant nutrient uptake, with would negative impact *P. xylostella* feeding rate. Insect root damage has been shown to reduce plant nutrient uptake in other plant-insect systems. Root herbivory by the weevil *Otiorhynchus sulcatus* induced a phosphorus deficiency in aboveground tissues, which reduced the growth of the leaf-feeding sawfly *Nematus olfaciens* (Johnson *et al.*, 2013). However, our results does not support this theory, as *P. xylostella* consumed leaf area was similar between insect treatments. Secondly, effects of root herbivory may have affected plant resource allocation leading to decreased insect feeding rate (Schultz *et al.*, 2013). For example, 18 hours of lubber grasshopper *Romalea guttata* feeding on maize, *Zea mays*, induced carbon re-allocation to roots (Holland *et al.*, 1996). We find a third explanation more likely, a systemic induction of defensive compounds from belowground feeding could have influenced leaf feeding (Papadopoulou & van Dam, 2017). Notably, sequential infestation with *P. xylostella* and *D. radicum* lower the survival of *D. radicum* adults on *B. oleracea* plants (Karssemeijer *et al.*, 2020). Combined with our findings this indicates a mutual negative effect of co-infestation for these herbivores on *B. oleracea*.

In a wider ecological context, an increasing amount of literature shows that rhizobacterial inoculation can alter insect herbivore behavior (Grunseich *et al.*, 2020). Rhizobacterial plant growth promotion that leads to larger and lusher plants could help insects to improve host location through visual cues. Despite a lack of knowledge of distant plant-host location behavioral changes due to rhizobacterial inoculation, several studies demonstrate altered insect preference after inoculation, perhaps by olfactory cues. For example, the root feeding larva of *Diabrotica speciosa* oriented toward non-inoculated roots instead of inoculated roots (Santos *et al.*, 2014). Aboveground insects may also show altered behavior. Rhizobacterial inoculation reduced cucumber beetle *Diabrotica undecimpunctata howardi* feeding on stems and cotyledons (Zehnder *et al.*, 1997). The lowered feeding correlated with a decrease in cucurbitacin synthesis in inoculated plants. As cucurbitacin is known as a feeding stimulant of *Diabrotica* beetles, lower amount of cucurbitacin can explain

the reduction in beetle feeding. However, other studies show no effect from rhizobacterial inoculation on insect preference. Rhizobacterial inoculation did not change the attraction of whitefly *Bemisia tabaci* adults when inoculated with the strain *Brevibacillus brevis* CBTC1 in genotypes of pepper *Capsicum annuum* (Chale-Carrillo *et al.*, 2016). Furthermore, rhizobacterial inoculation does not affect *M. brassicae* larval preference when *Arabidopsis* is inoculated by *P. simiae* WCS417r (Fernández de Bobadilla *et al.*, 2017). Whether these changes lead to insect biomass alterations or not, insect behavioral changes should be taken into account in rhizobacteria-insect studies.

In our study we demonstrated that rhizobacterial inoculation can promote plant growth and decrease the ingested amount of tissue consumed by a leaf feeder. We found that rhizobacterial soil addition did not differently modify plant resistance towards double herbivore infestation, compared to single herbivore infestation. Furthermore, we document that aboveground and belowground herbivory result in tissue-specific gene expression regulation.

Acknowledgements

The authors greatly appreciate support by the insect rearing staff of the Laboratory of Entomology for providing *Plutella xylostella*. The authors would like to thank the *Delia radicum* rearing team: Peter Karssemeijer, Kay Moisan and Max Wantulla. This work was funded by Wageningen University and private funding.

Author contributions

JF, JJAvL and MD conceived the study. JF and MP conducted the experiments. JF wrote the manuscript with the support of MD and JJAvL. All authors read and approved the manuscript.

Supplemental information

Table S1 Primers sequences.

Primers for *Brassica oleracea* target genes.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')	Tissue
LOX2	GCCATTGAGTTGACTCGTCC	GGATGCATGGCACTTAGTTGT	Leaf
MYC2	GGCTGGACCTACGCTATATTCTGG	AGAAAAACCACTCCGTATCCGT	Leaf/root
ORA59	AGGAAAGGGATAAGAGTGTGGCT	TCAAAGCTATCACCGGAGACTC	Leaf/Root
AOS	ACCGCTTGCGACTAGGGATC	CAAAGTCCTTACCGGCGCAC	Root
LOX6	AGGAGCTGCCAATTCGAAGC	CGCCTGTTCCAAAGTCATTCCA	Root

Primers for *Brassica oleracea* housekeeping genes.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')	Tissue
SAR1a	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT	Leaf
PER4	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC	Leaf
Btub	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC	Leaf/Root
Act-2	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG	Root

Chapter 6

Shoot and root insect herbivory change the plant rhizosphere microbiome and affect cabbage-insect interactions through plant-soil feedback

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Summary

Plant-soil feedback (PSF) has shown to influence plant-insect interactions. Here, we examined how insect herbivory and a beneficial rhizobacterium alter rhizosphere microbiota, subsequently influencing conspecific plants' growth and defense against root herbivores.

We conditioned soil with *Brassica oleracea* plants, infested with either the root-chewing herbivore *Delia radicum* (cabbage root fly), the leaf-chewing herbivore *Plutella xylostella* (diamondback moth), the phloem feeder *Brevicoryne brassicae* (cabbage aphid), and/or added the rhizobacterium *Pseudomonas simiae* WCS417r. We analyzed the rhizosphere microbial community, and in a second set of plants using the same soil, we assessed plant growth, expression of defense-related genes, and *D. radicum* performance.

The rhizosphere microbiome differed mainly between shoot and root herbivory treatments. Addition of *P. simiae* led to similar rhizosphere microbiome compared to that of the control plants. Soil conditioning affected plant shoot biomass, gene expression, and plant resistance against *D. radicum* in a treatment-specific manner. Plants had decreased shoot biomass when grown on conditioned compared to non-conditioned soil; *P. simiae*-amended soil caused the largest growth reduction.

We hypothesize that plant growth and defense may have been affected by changes in the abundance of specific rhizosphere microbes, rather than by the overall rhizosphere bacterial community.

Keywords: plant soil feedback, rhizosphere microbiome, plant defense, plant-mediated interactions, Brussels sprouts (*Brassica oleracea*), Cabbage root fly (*Delia radicum*), Diamondback moth (*Plutella xylostella*), rhizobacterium (*Pseudomonas simiae* WCS417r), Cabbage aphid (*Brevicoryne brassicae*)

Introduction

Plants are members of complex communities, in which they interact with a plethora of other organisms such as insects and microbes (van der Heijden *et al.*, 2008; Berendsen *et al.*, 2012; Stam *et al.*, 2014). Plant responses to the biotic or abiotic environment can affect many of these interactions and can shape the root and its microbiome (Sasse *et al.*, 2018; Stringlis *et al.*, 2019; Delory *et al.*, 2020). Shaping of the root-associated microbial community may impact future plants growing in the same soil. The net effect, either positive or negative, of all biotic and abiotic soil properties conditioned by plants that previously grew in that soil on plants subsequently growing in the same soil is called plant-soil feedback (PSF) (van der Putten *et al.*, 2013; Bennett & Klironomos, 2019). Plant-soil feedback can affect the performance of plants positively (Kulmatiski *et al.*, 2017) or negatively (Ma *et al.*, 2017; Lekberg *et al.*, 2018). Although an increasing number of studies focuses on the effects of PSF on plant growth, its effect on plant resistance is less explored, in particular plant defense against belowground insect herbivores (Hu *et al.*, 2018).

Plants possess interconnected hormonal signaling pathways that respond to insect herbivory. Plant defenses to insect herbivores are mainly regulated by the phytohormones salicylic acid (SA) and jasmonic acid (JA), but also other plant hormones such as abscisic acid (ABA) and ethylene (ET) are involved (Erb *et al.*, 2012b; Verma *et al.*, 2016). Aboveground, plants respond to herbivory by upregulating primarily SA- or JA-associated signaling depending on the species of the attacking insect. Generally, chewing insects induce JA production, whereas phloem-feeding insects induce SA biosynthesis (Erb *et al.*, 2012b; Stam *et al.*, 2014). In roots, JA is thought to be less inducible (Erb *et al.*, 2012a; Tytgat *et al.*, 2013), but increased levels do occur after herbivore attack (Erb *et al.*, 2009; Lu *et al.*, 2015; Karssemeijer *et al.*, 2020). SA may not serve the same function in root tissue as in shoot tissue (Erb *et al.*, 2012a; Lu *et al.*, 2015). These signaling pathways, and by extension plant defense relying on types and levels of secondary metabolites, can be modified by PSF (Ma *et al.*, 2017; Hu *et al.*, 2018; Zhu *et al.*, 2018; Bennett & Klironomos, 2019).

Plant-soil feedback starts with a plant influencing the surrounding soil, which impacts the root-associated microbiome (Wang *et al.*, 2019; Kostenko & Bezemer, 2020). Both shoot- and root herbivore feeding induces microbiome alterations through altered plant root exudation (Dawson *et al.*, 2004; Kostenko *et al.*, 2012; Kim *et al.*, 2016; Kong *et al.*, 2016; Ourry *et al.*, 2018). Herbivores can also influence the soil microbiome directly, for instance through insect frass that mixes with soil

(Poveda *et al.*, 2019), or indirectly through altering plant traits (Johnson *et al.*, 2016). The resulting changes in microbiome and soil properties affect the chemical composition of subsequently growing plants (Meiners *et al.*, 2017) which in turn can affect herbivorous insects (Kostenko *et al.*, 2012). Caterpillars of the cabbage moth *Mamestra brassicae* showed decreased performance when feeding on plants grown in soil conditioned by plants fed on by root-feeding wireworms *Agriotes lineatus*, compared to caterpillars feeding on plants grown in soil conditioned by caterpillar-infested plants (Kostenko *et al.*, 2012). Thus, herbivores can affect plant defense through PSF, but whether the underlying microbial changes are comparable between insect feeding guilds and feeding location, has received little attention.

Some root-associated bacteria are known to specifically enhance plant resistance and have been coined plant growth-promoting rhizobacteria (PGPR). These PGPR can induce systemic plant resistance (ISR), a mechanism that enhances resistance against a range of plant attackers (Pineda *et al.*, 2010; Pieterse *et al.*, 2014; Friman *et al.*, 2021b). These ISR-inducing bacteria can mediate plant-soil feedback. *Arabidopsis thaliana* recruited an assemblage of ISR-inducing microorganisms after infection with downy mildew, *Hyaloperonospora arabidopsidis*, which subsequently increased plant resistance of plants grown in the same soil against the same pathogen (Berendsen *et al.*, 2018). Although plant growth-promoting microbes are known to modulate plant resistance against insects (Pineda *et al.*, 2010), how these rhizobacteria subsequently affect plant defense against insects in conspecific plants growing in the same soil remains to be investigated.

Here, we studied how shoot- and root-feeding insect herbivores and beneficial rhizobacteria affect the rhizosphere microbiome. We then studied how these differences, through PSF, affect plant growth and defense against a root herbivore in a next set of plants grown on the same soil. We conditioned soil by growing a first set of *B. oleracea* plants induced by either the root chewer *Delia radicum*, the leaf chewer *Plutella xylostella,* the phloem feeder *Brevicoryne brassicae*, or by adding the growth-promoting and ISR-inducing PGPR *Pseudomonas simiae* WCS417r to the soil (Zamioudis *et al.*, 2013; Berendsen *et al.*, 2015). We quantified the rhizosphere microbial community after the conditioning phase. After removal of the plants, we used the conditioned soil mixed with sterilized soil (40:60 v/v), to grow a consecutive set of *B. oleracea* plants, for which we assessed plant performance, defense-related gene expression, and defense against the root herbivore *D. radicum*.

Materials and Methods

Plant growing conditions

Our study system consisted of *Brassica oleracea* L., a globally important cultivated crop plant. *Brassica oleracea* var. *gemmifera* cv. "Cyrus" seeds (Syngenta Seeds, the Netherlands) were germinated in a seeding tray with seedling soil (Supportive Information Table S1) in a greenhouse with 21±3°C and 16±3°C day and night temperatures respectively. Natural daylight was supplemented with 400 Watt metal halide lamps (200 µmol m-2 s-1 photosynthetically active radiation) when photosynthetic active radiation (PAR) dropped below 400 µmol m-2 s-1, in a 16:8 L:D cycle. After three days, plants were transplanted to 1 l pots containing potting soil (Supplemental Table S1) and grown in the greenhouse conditions for three weeks with identical settings as above at 60±10 % RH. Plants were watered three times per week until the top soil was moist. Plants were additionally fertilized twice per week with 50 ml of Hyponex solution (NPK = 7:6:19). Seedling and potting soil from the same batches were bagged and stored at 4°C for use in the feedback phase (Fig. **1**).

Insect rearing

Worldwide, the most important belowground feeding insect on *B. oleracea* is the specialist chewer cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae). The female flies deposit a cluster of eggs in the soil near the plant stem base. After hatching, the larvae feed on the primary root. The larvae leave the root to pupate in the soil and emerge later as adult flies. Experimental *D. radicum* larvae were reared on rutabaga roots (*B. napus* var*. napobrassica*) at 22±1 °C, 70% RH and a 16:8 L:D cycle. Adult flies were fed honey and a 1:1:1 mix of milk powder, sugar and yeast flakes. *Plutella xylostella* L. (Lepidoptera: Plutellidae) were reared on *B. oleracea* var. *gemmifera*. Second instar larvae were used in this experiment. *Brevicoryne brassicae* L. (Hemiptera: Aphididae) were reared on *B. oleracea* var. *gemmifera*, and wingless adults were used as inducers in the experiment. These insects were reared at 22±2°C, 70% RH and a 16:8 L:D cycle.

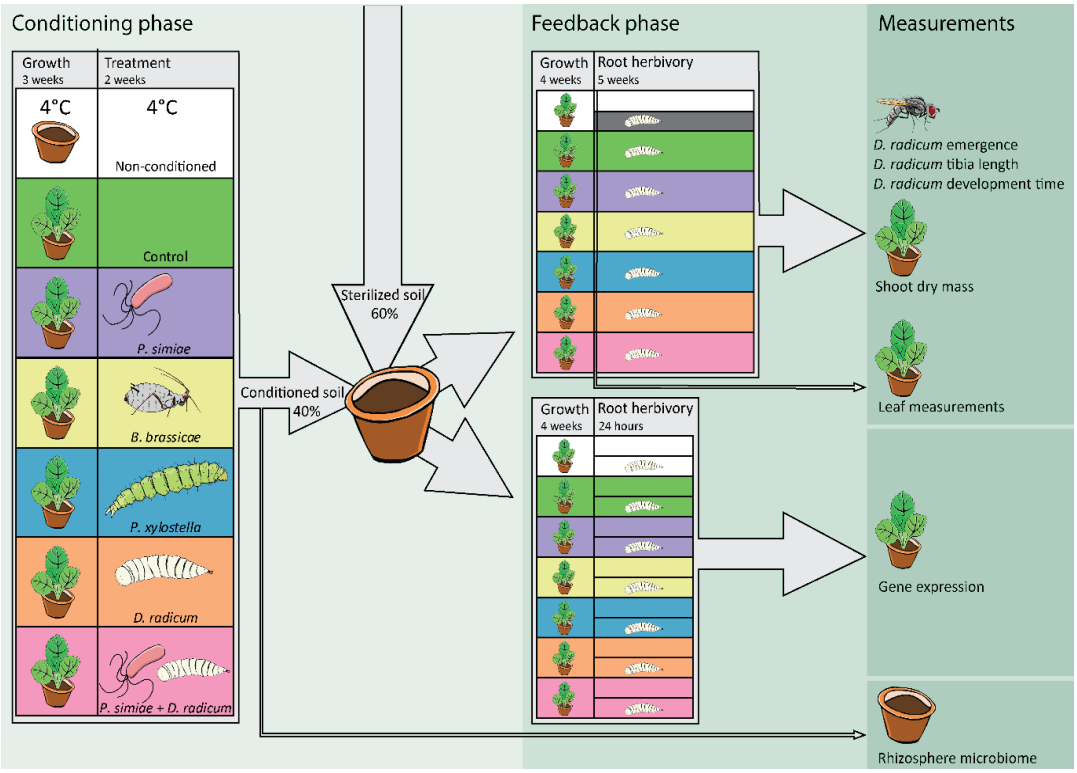


Figure 1 Overview of the experimental design.
Soil was conditioned by *Brassica oleracea* plants that, after three weeks of growth, were induced by six treatments represented by colored boxes in the conditioning phase. The treatments were uninfested plants (no herbivores, green), *Brevicoryne brassicae* (yellow), *Plutella xylostella* (blue), *Delia radicum* (orange), *Pseudomonas simiae* WCS417r (purple), *D. radicum* and *P. simiae* WCS417r (pink). Additionally, soil was stored at 4°C to be used as control in the feedback phase (white). After two weeks of induction, plants and insects were removed and rhizosphere microbiome samples were taken. The remaining soil of each treatment was mixed with sterilized soil (40:60 v/v). These soil mixes were used to grow two new sets of *B. oleracea* plants, one set was used for gene expression assessment (24 h post infestation) and the other set for plant and insect assessment (5 weeks post infestation). In the feedback phase, plants were exposed to *D. radicum* root herbivory, and insect performance was assessed, as well as plant performance and plant defense-related gene expression.

Pseudomonas simiae WCS417r growing conditions and solution preparation

The bacterial inoculum was prepared from *Pseudomonas simiae* WCS417r (formerly *P. fluorescens* (Berendsen *et al.*, 2015)) by incubating bacteria on King's B (KB) medium agar plates supplemented with rifampicin (25 µg ml^{-1}) for 48 h at 28°C. Cells were collected and suspended in sterilized 10 mM $MgSO_4$ solution. The suspension's optical density was adjusted to 1 x 10^9 CFU ml^{-1} (OD660 = 1.0).

Conditioning phase: induction with insects and rhizobacteria

After three weeks of growth, plants were infested with insects and/or exposed to *P. simiae* inoculum. Each treatment had 24 replicates divided over four trays with six plants placed in individual saucers, to prevent sharing water between plants. Treatments were *D. radicum*, *D. radicum* and *P. simiae* WCS417r, *P. xylostella*, *B. brassicae*, *P. simiae* WCS417r and untreated plants (Fig. **1**). For infestation with *P. xylostella* (L2) or *B. brassicae* (apterous adults), 10 individuals were carefully transferred to the fourth leaf counted along the stem from the stem base. To prevent insect contamination between treatments, the petiole of the infested leaf was wrapped in cotton wool, bagged in a net and fixed with a piece of metal wire. *Delia radicum* neonates were brushed on the carefully exposed stem base, just below soil level. For treatments receiving *P. simiae* WCS417r, bacterial suspension was applied next to the stem with a syringe. Each pot received 20 ml solution, which equals 2×10^{10} CFU, and 8×10^{7} CFU g^{-1} of soil.

Conditioning phase: soil and microbiome collection

Plants were exposed to insects and rhizobacterial inoculation for two weeks. Subsequently, aboveground plant parts and primary roots were removed from the soil. For soil microbiome analysis, around 3 g of secondary roots and root-attached soil were pooled from the six plants in each tray. Pooled roots were collected in 50 ml tubes containing 25 ml of sterilized buffer solution (6.33 g l^{-1} NaH_2PO_4 and 10.96 g l^{-1} NaH_2PO_4 x $2H_2O$). Tubes were vigorously shaken for 30 s, and centrifuged for 7 min at 4400 rpm. Supernatant was removed, as well as large chunks of root with sterilized tweezers. The soil slurry was transferred with a sterilized spoon into 1.5 ml tubes, and centrifuged for 5 min at 7800 rpm. Supernatant was removed and samples were then stored at -80°C. After taking microbiome samples, soils of all plants from the same treatment were homogenized by mixing by hand, using clean gloves for each treatment. For soils conditioned with plants infested with *D. radicum*, special care was taken to remove any larvae from the soil.

Feedback phase: setup and measurements

Soil from the conditioning phase was mixed with γ-irradiated soil (>25 KGray, Steris, Ede, the Netherlands) in a ratio of 40 % conditioned soil: 60 % sterilized soil (v/v). The soil mixture was divided over 1L pots. A non-conditioned soil treatment was added, consisting of pots containing a 40:60 mix of sterilized soil together with the soil stored as described above, to include a treatment consisting of soil with a microbiome similar to that of the soil used as starting material in the conditioning phase.

Brassica oleracea seeds were sown on seedling soil, stored at 4°C from the start of the experiment, to expose the seeds to a similar microbiome as the first set of plants. After three days, the seedlings were transplanted to the feedback phase pots. Plants were grown for 25 days under the same greenhouse settings as during the conditioning phase. After one week of plant growth the pots were provided with sticks to later support insect nets. Plants were divided into two sets, one for gene expression analysis after 24 h of exposure to *D. radicum* larvae and the other for assessing plant and *D. radicum* performance.

Feedback phase: plant and root herbivore performance

After four weeks of growth, plants were infested with 10 neonate *D. radicum* larvae. Half of the plants grown on non-conditioned soil were infested with larvae, to assess effects of *D. radicum* on plant performance. The larval infestation was performed as described above. For insect performance measurements, all plants were individually covered with a mesh bag 10 days after infestation. Plants were inspected daily for emerged *D. radicum* adults, which were then collected, frozen, and stored at -20°C. *Delia radicum* size was determined by measuring hind tibia length with a digital microscope (Dino-Lite Edge digital microscope, Taiwan). Developmental time was recorded as the time between larval infestation and adult emergence.

Plant performance in the feedback phase was assessed as leaf length and leaf width of the second leaf after three weeks of plant growth. As a proxy for plant size, average leaf size was calculated from these leaf measures using the following formula: length x width x leaf area coefficient. This coefficient was calculated by measuring the leaf area, width and length of ten *B. oleracea* leaves of similar size using LeafByte (Getman-Pickering *et al.*, 2020). Five weeks after infestation, the plant shoot was harvested and its biomass determined. Dry shoot biomass was recorded to the nearest 0.01 g (DK-6200-C-M, Allscales, USA) after drying at 105°C for 24 h.

Plant defense-related gene expression analysis

After four weeks of growth on conditioned soil, half of the plants were infested with 10 neonate *D. radicum* larvae (Fig. **1**), to assess plant defense gene expression under plant-soil feedback conditions. After 24 h of infestation, primary roots were harvested by uprooting the plants, cutting off secondary roots, and freezing the primary root directly in liquid nitrogen. One leaf disk from three leaves per plant was collected with a 1 cm diameter metal puncher. Samples were pooled for three plants, and

immediately frozen in liquid nitrogen to form one replicate. Samples were stored at -80°C.

Frozen samples were ground in liquid nitrogen, with a mortar and pestle for roots, or with a small pestle directly in the collection tube for leaves. Plant RNA was extracted with Isolate II Plant RNA kit (GCbiotech, the Netherlands) following the manufacturer's instructions, and converted to cDNA (SensiFAST, Bioline). Quantitative polymerase chain reaction (qPCR) analysis was performed to test transcript levels of genes of interest (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). The primer efficiency was calculated with qPCR by determining a standard curve with a dilution series. Reference genes *SAR1a*, *Btub*, *Act-2*, *PER4*, *GADHP* and *EF1a* were tested on 10 randomly selected samples from both roots and leaves to determine the optimal combination of reference genes using GeNorm (Vandesompele *et al.*, 2002) in qbase+ v.3.1 (Biogazelle, Zwijnaarde, Belgium). For roots, *Act-2* and *SAR1a* were used as reference genes, while for leaves *Btub* and *SAR1a* were used. We analyzed transcript levels in roots for *LOX6*, *MYB28*, *CYP81F1*, *MYB72* and *PDR9*, and in leaves for *LOX2* and *MYB28* (Supplemental Table **S2**). For *MYB72* and *PDR9*, two genes studied in *Arabidopsis* (At1g56160 and At3g53480, respectively), orthologous genes in *B. oleracea* were identified using the integrative orthology finder in PLAZA (van Bel *et al.*, 2018).

Delia radicum biomass assessment

One of the main challenges when working with *D. radicum* is the difficulty of assessing larval performance. The larvae are small and colorless, and during the first days of feeding they dig into the root, making it nigh impossible to find them back. To overcome this obstacle, we developed species-specific primers (Supplemental *Delia radicum* biomass assessment methods). These primers specifically target the 18S region of *D. radicum*, without amplifying non-targets such as those found in fungus gnats and nematodes which may occur in the experimental soil. We used these primers in the samples described above and normalized the quantity relative to the plant reference genes *Act-2* and *SAR1a* as a proxy of larval performance.

Soil microbiome analysis

Total genomic DNA (gDNA) from 0.25 ± 0.01 g of pooled rhizosphere soil was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The nucleic acid concentration and purity of samples were quantified with a spectrophotometer (DeNovix, Delaware, United States). For bacteria, the V4 region of the 16S gene was amplified using the 515F/806R primers

(Caporaso *et al.*, 2011) (Roche FastStart High Fi, 58°C, 26 cycles). For fungi, the ITS2 region was amplified using the fITS9/ITS4R primers (Ihrmark *et al.*, 2012) (Qiagen HotStarTaq, 52°C, 33 cycles). Microbial DNA was sequenced by Illumina MiSeq, 250bp paired-end, to a depth of 79,138 to 166,482 reads per sample. Amplification, library preparation and sequencing were performed by Génome Québec (Montreal, Canada).

Raw fastq files were processed using cutadapt (Martin, 2011) and the DADA2 pipeline (Callahan *et al.*, 2016). The code used for sample processing is available in the Supportive Information scripts. After processing, 62,735 to 97,854 bacterial reads and 47,339 to 98,457 fungal reads remained per sample. Taxonomy was assigned using the SILVA v138 database (Quast *et al.*, 2013) for bacteria and the UNITE v8.2 database (Nilsson *et al.*, 2018) for fungi. We filtered ASVs (Amplicon Sequence Variants) with too few occurrences using the effective sample approach in metagenomeSeq (Paulson *et al.*, 2013).

Statistical analysis

Statistical analysis was performed in R, version 4.0.0 (R Core Team, 2018), with R studio version 1.2.5042. For microbiome analysis, counts were normalized using metagenomeSeq. Principle component analysis (PCA) was performed using Bray-Curtis dissimilarity in phyloseq (McMurdie & Holmes, 2013). PERMANOVA was done with 99,999 permutations using Bray-Curtis dissimilarity with the adonis function (Oksanen *et al.*, 2007) post hoc analysis was performed in the RVAideMemoire package (Hervé, 2020). We tested whether differences in variance could have caused significant differences using permutest, which were non-significant for both bacterial and fungal analyses, indicating that the PERMANOVA results are valid. Differential ASVs were calculated using DESeq2 (Love *et al.*, 2014), by comparing each treatment to the control group with a false discovery rate of 0.05.

We used the packages tidyverse, lme4, emmeans, lmtest, lattice and fitdistrplus for plant and insect data (Zeileis & Hothorn, 2002; Sarkar, 2008; Bates *et al.*, 2015; Delignette-Muller & Dutang, 2015; Lenth *et al.*, 2018; Wickham *et al.*, 2019). The distribution of each dataset was explored with QQ-plots, histograms, Shapiro-Wilk test and the function descdist with 2000 bootstrapped values. Analysis of leaf length, plant shoot dry biomass and gene expression levels was performed with generalized linear models either using Gamma or Gaussian distributions. Development time, fly emergence and hind tibia length of *D*. *radicum* were analyzed by using generalized linear mixed models with Poisson, binomial and gamma distributions, respectively. Plant ID was used as a random factor to avoid pseudoreplication. Models were compared and

chosen based on Akaike Information Criterion (AIC) values. In the case of multiple fixed factors, the best model that included both factors ('soil treatment' and 'sex' or 'time') was chosen. Significance of fixed factors was assessed using the lrtest function.

Results

Insect herbivore-induced alterations in plant rhizosphere microbiome

Rhizospheres from plants in the conditioning phase were extracted and analyzed for bacterial and fungal communities. We found 1311 bacterial and 187 fungal Amplicon Sequence Variants (ASVs), the majority of which belong to the phyla Proteobacteria and Ascomycota, respectively (Supplemental Fig. **S1**).

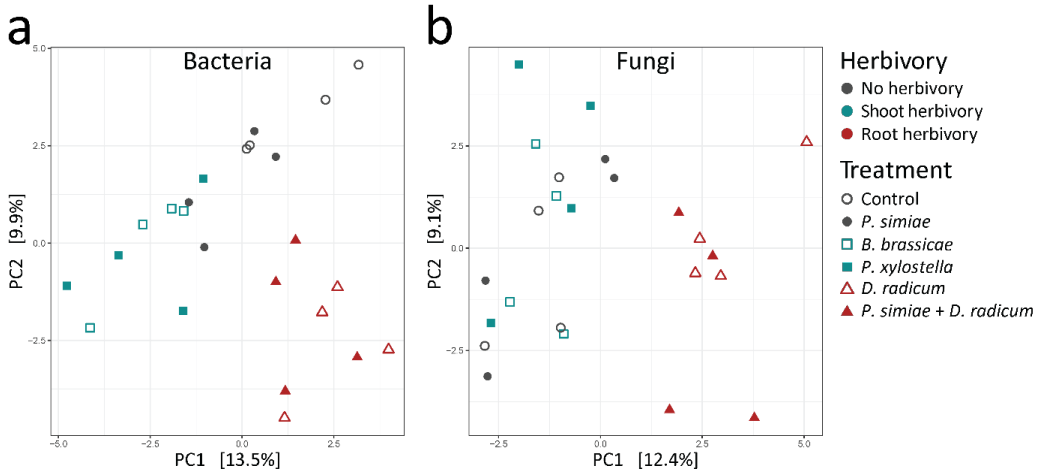


Figure 2 Principal Component Analysis (PCA) of bacterial (**a**) and fungal (**b**) rhizosphere communities. *Brassica oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. After two weeks, rhizosphere samples were collected and pooled from six plants. Bacterial 16S and fungal ITS2 regions were sequenced. Colors distinguish no herbivory, shoot or root herbivory; treatments are represented by shapes

Multivariate analysis revealed that microbial communities clustered by the presence and feeding location of inducing herbivores (Fig. **2**, Table **1**). The bacterial communities in rhizospheres of plants induced by root-feeding *D. radicum* clustered separately from plants induced by the shoot-feeding insects *B. brassicae* and *P. xylostella* and no herbivory (hereafter root herbivory, shoot herbivory, and no herbivory). These differences were confirmed by PERMANOVA (Table **1**), which showed that these three groups indeed differ in their bacterial communities (no herbivory – shoot herbivory: $F = 2.77$, $p < 0.001$, no herbivory – root herbivory: $F = 2.03$, $p < 0.001$, shoot herbivory – root

herbivory: F = 3.20, p < 0.001). Within these three groups, treatments did not differ from each other (Control – *P. simiae*: F = 1.17, p = 0.33, *B. brassicae* – *P. xylostella*: F = 0.84, p = 0.89; *D. radicum* – *P. simiae* + *D. radicum*: F = 1.04, p = 0.37). Fungi were also affected by the treatments, rhizosphere fungal communities from plants treated with root herbivory separated from the other samples on the first principal component (Fig. **2**, Table **1**). Rhizosphere fungal communities were strongly affected by root herbivory, and only slightly by shoot herbivory (no herbivory – shoot herbivory: F = 1.47, p = 0.01; no herbivory – root herbivory: F = 2.34, p < 0.001; shoot herbivory – root herbivory: F =2.48, p < 0.001). No differences were observed within the groups of shoot herbivory, root herbivory, or no herbivory (Control – *P. simiae*: F = 0.99, p = 0.64; *B. brassicae* – *P. xylostella*: F = 0.86, p = 0.77; *D. radicum* – *P. simiae* + *D. radicum*: F = 1.42, p = 0.09). Thus, feeding on either shoot or root tissue by herbivores appears to be an important factor in shaping the rhizosphere microbial community.

Table 1 Effects of treatment and herbivory on bacterial and fungal communities, where herbivory consisted of six treatments; *Brassica oleracea* plants were infested with (1) *Brevicoryne brassicae*, (2) *Plutella xylostella*, (3) *Delia radicum*, (4) inoculated with *Pseudomonas simiae* WCS417r, (5) infested with *D. radicum* and inoculated with *P. simiae*, or (6) uninfested plants used as control. These treatments were grouped into shoot, root-, or no herbivory to form the herbivory factor.

Variable	**Model type**	**Model**	***F***	**R^2**	***P*-value**
Bacterial communities	PERMANOVA	~Treatment	1.68	0.32	**< 0.001**
		~Herbivory	2.65	0.20	**< 0.001**
Fungal communities	PERMANOVA	~Treatment	1.49	0.29	**< 0.001**
		~Herbivory	2.09	0.17	**< 0.001**

To pinpoint specific changes caused by our treatments, we analyzed differentially abundant ASVs (Fig. **3**). For both bacteria and fungi, rhizospheres of plants treated with root herbivory were separated based on Euclidean distance from the shoot herbivory and no herbivory groups. Rhizospheres of plants treated with shoot herbivores also clustered in terms of bacteria, but not for fungal ASVs. For bacteria, ASVs were differentially abundant between rhizospheres of plants treated with *B. brassicae* and *P. xylostella* and control. For fungi, the largest number of ASVs were found for plants infested by *D. radicum* and *P. simiae* + *D. radicum*.

A cluster of five bacterial ASVs is present in rhizospheres of plants treated with root herbivory, while being absent in the control treatment; these include two members of the family Enterobacteriaceae, a *Klebsiella*, a *Pseudomonas*, and *Verruccomicrobiom spinosum*. Among the fungal ASVs, *Candida tropicalis* was strongly associated with rhizospheres of plants treated with *D. radicum*. Several ASVs, both bacteria and fungi, were affected only in rhizospheres of plants infested with *D. radicum* (without *P. simiae*); these ASVs are members of the bacterial families Nocardiaceae and Chitinophagaceae, genera *Bryobacter*, *Chryseobacterium* and *Roseiarcus*, and fungal order Helotiales, class Microbotryomycetes, and species *Candida palmioleophila* and *Coniochaeta fasciculata*.

Figure 3 (next page) Biclustered heatmaps showing differentially abundant bacterial (top) and fungal (bottom) ASVs. *Brassica oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. After two weeks, rhizospheres were collected in four samples, each pooled from six plants. Bacterial 16S and fungal ITS2 regions were sequenced. Differentially abundant ASVs were selected by DESeq2, with a threshold of FDR < 0.05 difference between treatment and control. Colored circles right of the heatmaps show whether an ASV is significantly different between that treatment and control. Clustering by shoot and root herbivory and treatment is based on Euclidean distance. Colors show $\log_2$ (normalized count +1).


Bacterial 16S
Herbivory
Treatment
P. simiae
B. brassicae
P. xylostella
D. radicum
P. simiae + D. radicum
p_Proteobacteria | g_Rhodanobacter
p_Proteobacteria | f_Xanthobacteraceae
p_Proteobacteria | g_Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium
p_Actinobacteriota | f_Streptomycetaceae
p_Actinobacteriota | g_Nocardia
p_Planctomycetota | g_Paludisphaera
p_Actinobacteriota | f_67−14
p_Planctomycetota | f_Pirellulaceae
p_Proteobacteria | g_Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium
p_Proteobacteria | g_Burkholderia−Caballeronia−Paraburkholderia
p_Proteobacteria | g_Burkholderia−Caballeronia−Paraburkholderia
p_Proteobacteria | g_Rhodanobacter
p_Proteobacteria | f_Rhodanobacteraceae
p_Proteobacteria | f_Enterobacteriaceae
p_Proteobacteria | g_Klebsiella
p_Proteobacteria | g_Pseudomonas
p_Proteobacteria | f_Enterobacteriaceae
p_Verrucomicrobiota | g_Verrucomicrobium | s_spinosum
p_Proteobacteria | g_Micropepsis
p_Planctomycetota | f_Gemmataceae
p_Proteobacteria | f_Rhizobiales Incertae Sedis
p_Proteobacteria | g_Pseudaminobacter
p_Proteobacteria | g_Herbaspirillum
p_Proteobacteria | g_Bordetella
p_Verrucomicrobiota | f_Pedosphaeraceae
p_Proteobacteria | g_Micropepsis
p_WPS−2
p_Verrucomicrobiota | f_Pedosphaeraceae
p_Proteobacteria | g_Sphingomonas
p_Bacteroidota | g_Puia
p_Bacteroidota | g_Mucilaginibacter
p_Actinobacteriota | o_Micrococcales
p_Bacteroidota | f_Chitinophagaceae
p_Actinobacteriota | g_Streptomyces
p_Proteobacteria | g_Sphingomonas
p_Proteobacteria | g_Roseiarcus | s_fermentans
p_Planctomycetota | g_Candidatus Nostocoida
p_Bacteroidota | g_Flavobacterium
p_Proteobacteria | g_Azospirillum
p_Proteobacteria | g_Hyphomicrobium
p_Verrucomicrobiota | f_Verrucomicrobiaceae
p_Bacteroidota | g_Parafilimonas | s_terrae
p_Proteobacteria | g_Alkanibacter
p_Bacteroidota | g_Cytophaga
p_Proteobacteria | f_Morganellaceae
p_Cyanobacteria | c_Sericytochromatia
p_Planctomycetota | g_Aquisphaera
p_Firmicutes | g_Clostridium sensu stricto 13
p_Acidobacteriota | g_Granulicella | s_paludicola
p_Acidobacteriota | g_Bryobacter
p_Bacteroidota | f_Chitinophagaceae
p_FCPU426
p_Verrucomicrobiota | g_Lacunisphaera | s_anatis
p_Bacteroidota | g_Pedobacter
p_Bacteroidota | g_Chryseobacterium
p_Bacteroidota | g_Mucilaginibacter
p_Proteobacteria | g_Pseudomonas
p_Proteobacteria | f_Moraxellaceae
p_Bacteroidota | g_Cytophaga
p_Actinobacteriota | f_Nocardiaceae
p_Proteobacteria | g_Roseiarcus
Herbivory
No herbivory
Shoot herbivory
Root herbivory
Treatment
Control
P. simiae
B. brassicae
P. xylostella
D. radicum
P. simiae + D. radicum
14
12
10
8
6
4
2
0
log2 (normalized counts + 1)
Fungal ITS
Herbivory
Treatment
P. simiae
B. brassicae
P. xylostella
D. radicum
P. simiae + D. radicum
p_Ascomycota | g_Coniochaeta | s_fasciculata
p_Ascomycota | o_Helotiales
p_Basidiomycota | g_Clitopilus
p_Basidiomycota | o_Sebacinales
p_Basidiomycota | o_Sebacinales
p_Ascomycota | g_Hawksworthiomyces | s_lignivorus
p_Basidiomycota | c_Microbotryomycetes
p_Ascomycota | g_Oidiodendron
p_Ascomycota | g_Rasamsonia | s_byssochlamydoides
p_Ascomycota | g_Penicillium
p_Ascomycota
p_Ascomycota | g_Goidanichiella | s_barronii
p_Ascomycota | g_Talaromyces | s_radicus
p_Ascomycota | o_Magnaporthales
p_Chytridiomycota | g_Kappamyces
p_Ascomycota
p_Ascomycota | g_Pseudogymnoascus
p_Ascomycota | g_Candida | s_palmioleophila
p_Ascomycota | g_Candida | s_tropicalis
p_Ascomycota | g_Trichoderma | s_hamatum
p_Ascomycota | g_Candida | s_tropicalis
p_Ascomycota | g_Candida | s_tropicalis
p_Ascomycota | g_Candida | s_tropicalis

Further, a group of highly abundant bacterial ASVs were changed in rhizospheres of *P. xylostella* and *B. brassicae*-treated plants compared to control plants. For instance, a member of the genus *Rhodanobacter* was the most abundant ASV in the overall bacterial community, and it was reduced from an average of 3700 normalized counts (4.8% relative abundance) in the rhizosphere of control plants, to 2600 (3.9% relative abundance) and 2500 (3.8% relative abundance) in rhizospheres of *P. xylostella* and *B. brassicae*-treated plants, respectively. Interestingly, several bacterial ASVs were depleted specifically in rhizospheres of *B. brassicae*-treated plants compared to rhizosphere of control plants, including members of the genera *Flavobacterium*, *Azospirillum*, *Hyphomicrobium*, *Alkanibacter*, *Cytophaga*, and the species *Parafilimonas terrae*.

Rhizospheres of plants inoculated with *P. simiae* only differed from those of control plants in four bacterial ASVs, while eight fungal ASVs were affected. Of those four bacterial ASVs in rhizospheres of *P. simiae*-inoculated plants, one is a *Pseudomonas* fully matching *P. simiae* WCS417r through a BLAST search. However, the sequenced 16S fragments are identical to many strains in the related group *Pseudomonads*, therefore we cannot verify that these fragments are explicitly from the strain used in the experiment. Two fungal ASVs, *Hawksworthiomyces lignovirorous* and *Trichoderma hamatum*, are specifically depleted in rhizospheres of plants inoculated with *P. simiae*.

Table 2 Effects of the factors soil treatment, root herbivory and sex on *Delia radicum* performance variables, and effects on plant performance and gene expression of *Brassica oleracea*.

Variable	Model type	Model	Factor	X^2	df	*P*-value
Delia radicum emergence	GLMM Binomial	~ Soil treatment + PlantID*	Soil treatment	25.62	6	**<0.001**
Delia radicum tibia length	LMM Normal	~ Soil treatment + Sex + PlantID*	Soil treatment	14.18	6	**0.028**
			Sex	68.87	1	**<0.001**
Delia radicum 18S	GLM Gamma	~ Soil treatment	Soil treatment	15.56	6	**0.016**
Leaf area	GLM Gamma	~ Soil treatment	Soil treatment	383.57	6	**<0.001**
Plant dry mass	GLM Gamma	~ Soil treatment	Soil treatment	336.44	7	**<0.001**
Root *LOX6*	GLM Gamma	~ Soil treatment + Root herbivory	Soil treatment	6.13	6	0.408
			Root herbivory	33.27	1	**<0.001**
Root *MYB28*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	1.82	6	0.935
			Root herbivory	125.31	1	**<0.001**
			Interaction	27.84	6	**<0.001**
Root *CYP81F4*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	1.33	6	0.97
			Root herbivory	105.76	1	**<0.001**
			Interaction	15.09	6	0.02
Root *MYB72*	GLM Gamma	~ Soil treatment + Root herbivory	Soil treatment	20.27	6	**0.002**
			Root herbivory	0.57	1	0.451
Root *PDR9*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	31.83	6	**<0.001**
			Root herbivory	20.91	1	**<0.001**
			Interaction	23.71	6	**<0.001**
Leaf *LOX2*	LM Normal	~ Soil treatment × Root herbivory	Soil treatment	6.75	6	0.344
			Root herbivory	37.86	1	**<0.001**
			Interaction	14.91	6	**0.021**
Leaf *MYB28*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	30.26	6	**<0.001**
			Root herbivory	3.46	1	0.063
			Interaction	8.98	6	0.175

(G)L(M)M: (Generalized) Linear (Mixed) Model.
*PlantID was included in the models as a random factor to avoid pseudoreplication as multiple flies emerged from each plant.

Plant-soil feedback effects on plant performance

To assess whether plant-soil microbiome alterations affected consecutively growing plants and their insect resistance, *B. oleracea* plants were grown in the same soil previously conditioned by conspecific plants exposed to different treatments. Plant leaf area was measured for the second leaf counted from the bottom along the stem before infestation after four weeks of plant growth, and shoot dry mass was measured after five weeks after infestation. The plants received regular and similar amounts of plant fertilizer. The surface area of the second leaf was affected by soil conditioning (Fig. **4a**): plants grown on conditioned soil had smaller leaves. Plant shoot dry mass was also affected by soil conditioning (Fig. **4b**), where dry shoot biomass of plants grown on conditioned soil was lower compared to plants grown on non-conditioned soils. Plants grown on soil conditioned by plants inoculated with *P. simiae* were smaller compared to plants grown on soil conditioned by control plants. Plants grown on soil conditioned by plants treated with *P. xylostella* were larger, both in terms of leaf size and biomass.

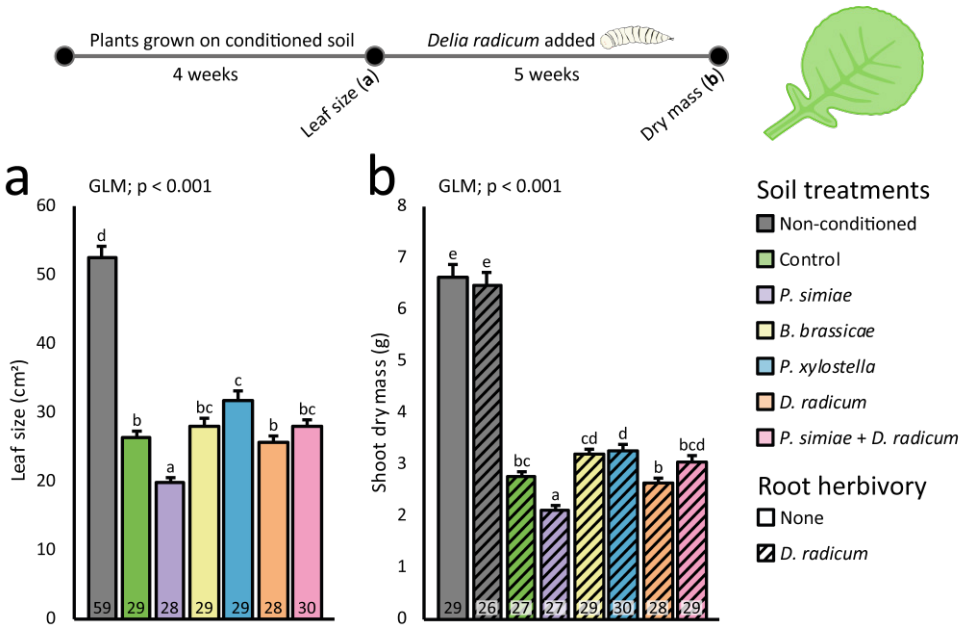


Figure 4 Size of leaf number 2 counted from the bottom on the stem (**a**) and dry shoot biomass after *Delia radicum* infestation (**b**) of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, leaf size of these new plants was quantified before infestation with insect herbivores (**a**). After five weeks of infestation, the plants were harvested, and dry shoot biomass was measured (**b**). All plants in the feedback phase, and a subset of plants on non-conditioned soil, were induced with 10 *D. radicum* larvae after three weeks of growth. Numbers in bars represent the number of included plants, bars with different letters within a panel are significantly different from one another (Tukey's HSD, α = 0.05), and bars show mean + SE. GLM; Generalized Linear Model. Bars represent means + SE.

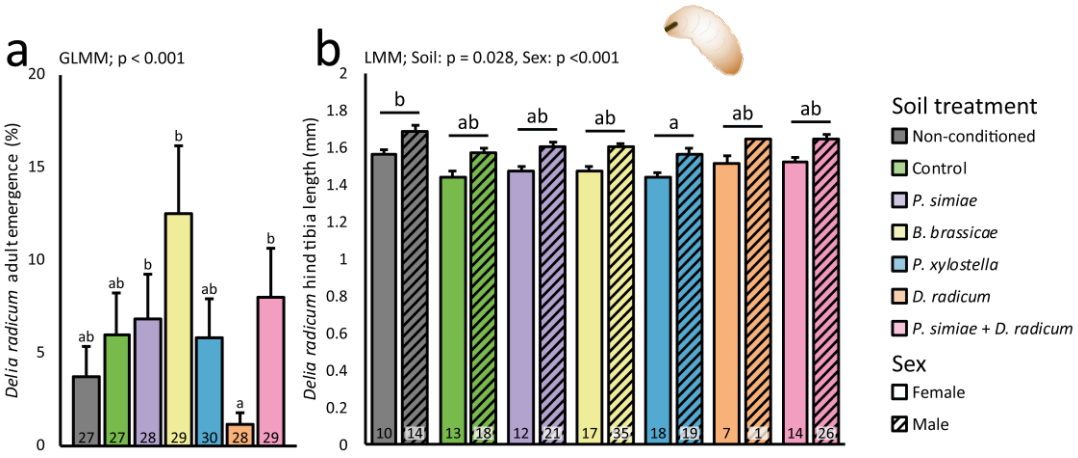


Figure 5 *Delia radicum* adult emergence (**a**) and hind tibia length (**b**) on *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, these new plants were infested with *D. radicum* larvae, and emerging flies counted and their hind tibia length measured. Numbers in bars represent the number of plants (**a**) or flies (**b**), bars with different letters are significantly different from each other (Tukey's HSD, α = 0.05), and bars show mean + SE. Due to low sample size, no SE could be calculated for males in the *D. radicum* treatment (orange striped bar). Soil; Soil conditioning treatment, (G)LMM; (Generalized) Linear Mixed Model.

Plant-soil feedback effects on Delia radicum performance

To unravel belowground plant resistance in a plant-soil feedback context, we infested *B. oleracea* plants grown in conditioned soils with *D. radicum* larvae. Adult fly emergence from soil was assessed and tibia length was measured as a proxy for fly body size. Overall, *D. radicum* adult emergence was low in the experiment, on average 11.4% (N_{total} = 1970) of larvae developed into adults. In addition to these performance measures, in the plants used for gene expression analysis we examined insect performance through analysis of *D. radicum* 18S ribosomal RNA.

Emergence of *D. radicum* was affected by soil conditioning in a treatment-specific way (Fig. **5a**). Fewer flies emerged from plants grown on soil conditioned by plants infested by *D. radicum* compared to plants grown on soils conditioned by plants treated with *B. brassicae* or *D. radicum* together with *P. simiae*. Tibia length of adult flies was affected by soil conditioning (Fig. **5b**). Flies with smaller tibia length emerged from plants grown on soil conditioned by plants infested with *P. xylostella* compared to flies that emerged from plants grown on non-conditioned

soil. Fly development time was similar for all treatments (data not shown).

In the set of plants used for gene expression analysis 24 h post infestation, we quantified *D. radicum* 18S ribosomal RNA relative to plant reference genes as a proxy of *D. radicum* performance (Fig. **6f**). Relative quantities of *D. radicum* 18S were affected by soil conditioning treatments. This analysis supports the observation that *D. radicum* performance was reduced in plants grown on soil conditioned by *D. radicum* compared to plants grown on non-conditioned soil (Tukey's HSD; $z = 3.59$, $p < 0.05$) or soil conditioned by control plants (Tukey's HSD; $z = 2.88$, $p = 0.061$). Taken together, the results show that *D. radicum* was negatively affected when feeding on plants that had been growing in soil conditioned by plants also exposed to feeding by conspecific larvae.

Gene expression in response to Delia radicum infestation and plant-soil feedback treatments

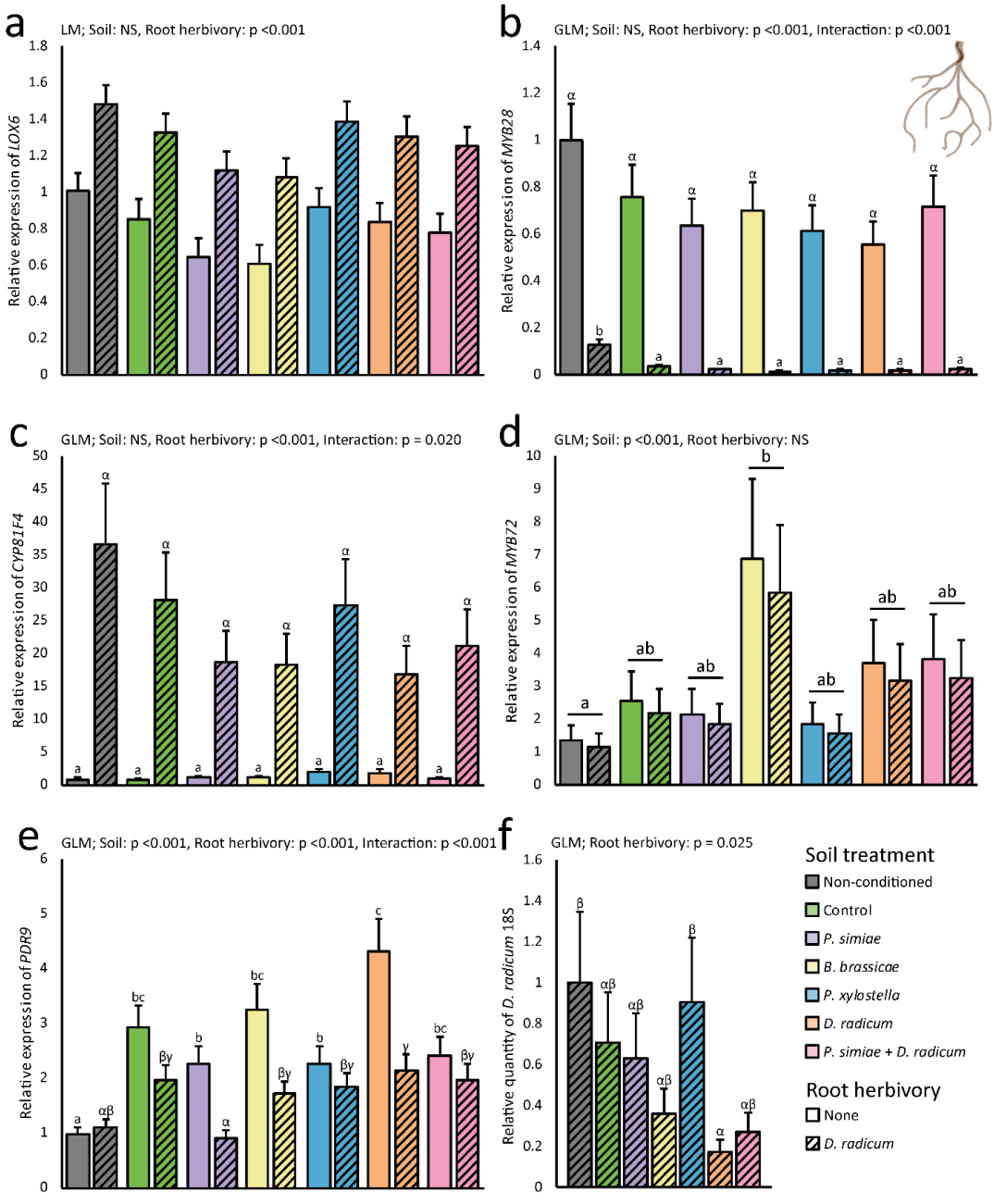
We assessed primary root defense responses to herbivory by *D. radicum* in plants grown on conditioned and non-conditioned soil, measured after 24 h of *D. radicum* infestation of the primary root. Expression in the roots of *LOX6*, a gene involved in JA biosynthesis, was induced by *D. radicum* regardless of soil conditioning (Fig. **6a**). Transcript levels in the roots of *MYB28*, involved in aliphatic GSL biosynthesis, were downregulated by *D. radicum* infestation (Fig. **6b**). The soil conditioning treatments did not affect root *MYB28* expression, but there was an interaction effect between *D. radicum* infestation and soil conditioning. When infested with *D. radicum*, transcript levels of *MYB28* were lower in plants grown on conditioned soils compared to non-conditioned. In contrast to *MYB28* downregulation by *D. radicum* infestation, mRNA levels of *CYP81F4*, encoding an enzyme involved in indole GSL biosynthesis, were strongly upregulated by infestation. Type of soil conditioning did not influence *CYP81F4* transcript levels, but there was an interaction between *D. radicum* and soil conditioning (Fig. **6c**).

Expression of root *MYB72*, a transcription factor involved in induced systemic resistance and iron acquisition (van der Ent *et al.*, 2008; Palmer *et al.*, 2013), was affected by soil conditioning in a treatment specific way, but not by *D. radicum* infestation (Fig. **6d**). Transcript levels of *PDR9*, a gene encoding a transporter involved in root exudation of coumarins, were affected by both soil treatment and *D. radicum* infestation, and there was an interaction between soil treatment and *D. radicum* infestation (Fig. **6e**). When no *D. radicum* was present,

expression of *PDR9* was upregulated in primary roots of plants subjected to all soil conditioning treatments compared to plants grown on non-conditioned soil, especially when soil was conditioned by plants infested with *D*. *radicum*. This effect was attenuated upon *D*. *radicum* infestation, in which case transcript levels of *PDR9* did not differ between soil conditioning treatments.

Leaf transcript levels of *LOX2*, a marker gene for JA biosynthesis expressed in the shoot, were increased by root herbivory but not by soil conditioning; there was a significant interaction effect between soil conditioning and root herbivory (Fig. **S3a**). *MYB28* transcript levels in leaves were affected by soil conditioning treatments (Fig. **S3b**), but not by *D. radicum* infestation.

Figure 6 (next page) Relative gene expression of *LOX6* (**a**), *MYB28* (**b**), *CYP81F4* (**c**), *MYB72* (**d**), *PDR9* (**e**) and *Delia radicum* 18S (**f**) in primary roots of *Brassica oleracea* plants grown in conditioned soil by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *D. radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, half of these plants were infested with *D. radicum* (x-axis). All bars are set relative to the gene expression levels in primary roots of plants of non-infested plants grown in non-conditioned soil (grey bar). Bars with different letters are significantly different from one another, within control (Roman alphabet) or *D*. *radicum* infested (Greek alphabet) samples (Tukey's HSD, $\alpha = 0.05$), and bars show mean + SE. Soil; Soil conditioning treatment, Delia; *D*. *radicum* treatment, NS; Not significant, (G)LM; (Generalized) Linear Model. N = 3 or 4 replicates of 3 pooled plants.


a
LM; Soil: NS, Root herbivory: p <0.001
Relative expression of LOX6
b
GLM; Soil: NS, Root herbivory: p <0.001, Interaction: p <0.001
Relative expression of MYB28
c
GLM; Soil: NS, Root herbivory: p <0.001, Interaction: p = 0.020
Relative expression of CYP81F4
d
GLM; Soil: p <0.001, Root herbivory: NS
Relative expression of MYB72
e
GLM; Soil: p <0.001, Root herbivory: p <0.001, Interaction: p <0.001
Relative expression of PDR9
f
GLM; Root herbivory: p = 0.025
Relative quantity of D. radicum 18S
Soil treatment
Non-conditioned
Control
P. simiae
B. brassicae
P. xylostella
D. radicum
P. simiae + D. radicum
Root herbivory
None
D. radicum

Discussion

Our study shows that the plant root microbiome changes after insect attack, and that plant growth and defense are influenced *via* PSF. Our data show that the bacterial rhizosphere community is differentially affected by shoot and root herbivory, whereas the fungal rhizosphere community is mostly affected by root herbivory. Although previous research shows that plant defense against shoot-feeding insects can be altered through PSF (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013; Kos *et al.*, 2015a; Kos *et al.*, 2015b; Hu *et al.*, 2018; Pineda *et al.*, 2020), we here show novel evidence that the root-feeding insect *D. radicum* is negatively affected by conspecific feeding through PSF.

We observed that herbivore feeding on the root or the shoot influenced the rhizosphere microbial community. The multivariate analysis revealed that bacterial rhizosphere communities were separated into three groups: plants with shoot herbivory, with root herbivory and non-infested plants. We further observed that the fungal rhizosphere community was similar between plants fed on by shoot-feeding insects and control plants, but was different from the fungal community of plants with root-feeding *D. radicum*. A previous study showed that *D. radicum* herbivory led to only minor changes in fungal community, but caused major changes in both endo- and rhizosphere bacterial communities of oilseed rape, *B. napus* (Ourry *et al.*, 2018). Interestingly, our results show that *D. radicum* herbivory strongly increased the abundance of the soil yeast *Candida tropicalis*, of which plant-growth promoting strains are known (Amprayn *et al.*, 2012). Furthermore, rhizospheres of *D. radicum*-infested plants showed an accumulation of several bacteria (Enterobacteriaceae, *Klebsiella*, and *Pseudomonas*) that may be associated with the *D. radicum* gut microbiome (Lukwinski *et al.*, 2006; van den Bosch & Welte, 2020). This finding hints at the interesting possibility of direct interactions between the microbiomes of the plant and the root herbivore gut. For shoot feeding insects, previous studies showed both response of the rhizosphere community, and no response. In line with our results, some studies show an effect of herbivory on the rhizosphere community (Yang *et al.*, 2011; Lee *et al.*, 2012; Bezemer *et al.*, 2013; Kong *et al.*, 2016; Malacrinò *et al.*, 2020; Zytynska *et al.*, 2020). Other studies report similar rhizosphere microbiomes for shoot-herbivore-infested and non-infested plants (O'Brien *et al.*, 2018; Malacrinò *et al.*, 2020). The variation seen in the literature regarding rhizosphere microbiome responses to shoot herbivory could be explained by factors such as plant- and insect-specific responses, or different bulk soil bacterial communities in the starting soil.

We found that inoculation with the rhizobacterium *P. simiae* did not affect overall microbial communities in the rhizosphere. Although there are studies that find an altered root community after addition of rhizobacteria, others report no effects (Herschkovitz *et al.*, 2005; Gadhave *et al.*, 2018; Wang *et al.*, 2018; Zytynska *et al.*, 2020). Even though the microbial community composition was not affected by the addition of *P. simiae* WCS417, the abundance of several distinct species was changed. It has been demonstrated that only a set of three bacterial soil species are sufficient to increase resistance in *A. thaliana* against a foliar fungal pathogen (Berendsen *et al.*, 2018). Thus, changes in a few species have the potential to have large consequences for the plant.

In the feedback phase of our experiment, plants showed treatment-dependent responses in plant performance when grown on conditioned soils. Shoot herbivory by *P. xylostella* on plants during the conditioning phase led to increased growth of plants in the feedback phase, compared to plants grown in soil conditioned by plants without herbivores. Hence, herbivory can affect plants over growing periods *via* soil-mediated effects. Several PSF mechanisms could have contributed to our results. Fresh litter, such as fine roots, can stimulate the microbial activity (Fontaine et al. 2003), but can also negatively affect plant growth through release of phytotoxic (allelopathic) and autotoxic compounds when decomposing (Bonanomi *et al.*, 2006). Extracellular self-DNA (eDNA) is also released from decomposing tissue, and can exert plant growth inhibition on grasses, forbs and *A. thaliana in vitro* (Mazzoleni *et al.*, 2015). These PSF mechanisms are likely to have contributed to our results, as root fragments were present in the soil we transferred.

Surprisingly, the performance of *B. oleracea* was drastically decreased when grown in soil on which previously growing plants were inoculated with *P. simiae*, compared to the other soil conditioning treatments. Although this PGPR strain is usually considered a beneficial rhizobacterium when applied to plants, such as *B*. *oleracea* (Friman *et al.*, 2021a), our results suggest that this beneficial effect may not be maintained through PSF. Notably, there are reports of rhizobacteria causing effects varying from plant growth promotion to inhibition, depending on e.g. phosphate availability or rhizobacterial population density (Ciccillo *et al.*, 2002; Morcillo *et al.*, 2020). However, as we assume the nutrient availability was sufficient for the experimental plants due to regular fertilization in our experiments, we instead propose that changes in the microbiome underlie the reduction in growth. For example, *Trichoderma hamatum* was absent in rhizospheres of *P. simiae*-induced plants while it was present in the other treatments. This species is a known growth-promoting fungal species in *e.g.* pepper (Mao *et al.*, 2020).

In this way, the addition of *P. simiae* may have suppressed other beneficial microbes in the rhizosphere, leading to a net negative growth effect. Interestingly, herbivory by *D. radicum* together with *P. simiae* inoculation of the plants during the conditioning phase restored plant biomass to a certain degree in the feedback phase. Evidently, *D*. *radicum* has a more dominant impact on the rhizosphere microbiome compared to addition of *P*. *simiae* WCS417r.

Root herbivory by *D. radicum* during the conditioning phase led to lower performance of *D. radicum* in the feedback phase, in line with previous studies that demonstrated alteration of plant resistance against insects through PSF (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013; Kos *et al.*, 2015a; Kos *et al.*, 2015b; Hu *et al.*, 2018; Pineda *et al.*, 2020). The overall *D. radicum* adult emergence in our experiment was low compared to other studies using similar methods (Soler *et al.*, 2007; van Geem *et al.*, 2015; Karssemeijer *et al.*, 2020). Although this is a limitation of our study, we reinforced our results by the quantification of *D. radicum* 18S ribosomal RNA after 24 h of feeding, a novel method to quantify root fly larval performance *in planta*. This method confirmed a lower performance of *D. radicum* on plants in the feedback phase growing in soil conditioned with *D. radicum*-infested plants. Performance of *D. radicum* may have been affected by a change in plant defense, or by a direct influence of the soil microbiome. Indeed, Lachaise *et al.* (2017) found that differences in the soil microbiome affected *D. radicum* performance. *Delia radicum* infestation was previously shown to increase the abundance of *Bacillus* and *Paenibacillus* in the rhizosphere, which could have entomopathogenic properties (Ourry *et al.*, 2018). These species were not differentially affected in our study, perhaps due to different plant growing substrates. Without isolating these bacteria from the rhizosphere and testing their effects on the plant and the root herbivore larvae, we can only speculate about the underlying mechanisms.

In roots, most defense markers we studied were not affected by soil conditioning treatments, and thus they do not explain the difference in insect performance. However, since only a single time point was studied, we cannot rule out that soil microbes may have primed defense against *D. radicum*, leading to a faster defensive response. Indeed, two genes involved in ISR, *MYB72* and *PDR9*, were affected by soil conditioning treatments. Although these genes have mainly been studied in *A. thaliana,* we found that the expression of their orthologues in *B. oleracea* was changed by soil conditioning. The transcription factor *MYB72* has been identified as a key regulation node in *A. thaliana* roots in iron uptake and communication with the beneficial rhizobacterium *P. simiae* WCS417r (Verhagen *et al.*, 2004) and was later verified to play a central

role in rhizobacterial ISR (van der Ent *et al.*, 2008). This transcription factor regulates the expression of genes involved in the shikimate, phenylpropanoid and nicotianamine biosynthesis pathways, including genes leading to the production and exudation of coumarins (Zamioudis *et al.*, 2014). These coumarins, in particular scopoletin, are exuded from the roots by the transporter *PDR9*, where they play a dual role in both the plant response to iron deficiency and influencing the rhizosphere microbiome (Stringlis *et al.*, 2018; Stringlis *et al.*, 2019). This could be an indication that ISR plays a role in PSF. Interestingly, transcript levels of *LOX2* and *MYB28* in leaves were affected by soil conditioning treatments, a result which is in line with previous studies that found a link between shoot defense and plant-soil feedback (Hu *et al.*, 2018). This again underlines that defense signaling in shoot and root is fundamentally different (Johnson *et al.*, 2016).

In conclusion, our study demonstrates that shoot and root herbivory lead to separate plant rhizosphere microbe communities. Furthermore, we show that inoculation of *P. simiae* to the soil has limited effects on the rhizosphere microbial community. Through PSF, plant performance and defense is altered in a treatment-dependent way for *B. oleracea* plants growing in soil conditioned by conspecific plants. The results presented here suggest that changes in the abundance of specific microbes, rather than the overall microbiome, may be more important for plant performance and defense. Finally, our results underline the complexity of the interplay between rhizosphere microbes, insects, and plants.

Acknowledgments

The authors would like to thank Laura Winzen, Camilo Rivera Arrivillaga, Viktoria Hartan and Giulia Berni for experimental assistance, and Pedro Beschoren da Costa for discussions on data analysis. We would like to thank the Laboratory of Entomology insect rearing team, and Kay Moisan and Max Wantulla for help in rearing cabbage root flies, and Sean Geurts for growing plants. We thank Yidong Wang for drawing the insects and plants in our illustrations. This work was supported by Wageningen University and private funding.

Authors contributions

PK and JF designed the study. JH together with JF and PK conducted the experiments and performed molecular analysis. JH, JF and PK analyzed the data with assistance of JJAvL and MD. KdK and PK developed the *Delia radicum* 18S primers. JF and PK wrote the manuscript, with the help of JJAvL and MD.

Supplemental information

Table S1 Soil properties of potting soil.

Soil pH: 5.70 EC: 0.80 EN-factor: 1.23 HWW: 180

Hortiklei!	40,610 kg
MD Zweeds veenmosveen	0,200 EN-m³
Baltisch veen middel	0,400 EN-m³
Tuinturf normaal	0,400 EN-m³
Dolokal Extra potgrond (bulk)	3,820 kg
PG-mix 15-10-20	0,810 kg

Table S2 Primers for target genes in *Brassica oleracea*.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')	Tissue
LOX2	GCCATTGAGTTGACTCGTCC	GGATGCATGGCACTTAGTTGT	Leaf
LOX6	AGGAGCTGCCAATTCGAAGC	CGCCTGTTCCAAAGTCATTCCA	Root
CYP81F1	TGTGTCAGAAACGTTCAGGCT	ATGGCACGTCGTATCCTCCG	Root
MYB28	CGGGAGAGATGAGCACAATACG	CAGCCCTCGAAGTTTCCTATCA	Leaf/Root
MYB72	AAACAAGTGGTCAAAGATCGCG	ACACTCATCTCAAGAAACGACT	Root
PDR9	ATTCCACCACCTTCTATGCCG	ACTTGGTTGTATCTGGCTCC	Root

Primers for reference genes in *Brassica oleracea*.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')	Tissue
SAR1a	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT	Leaf/Root
Btub	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC	Leaf/Root
Act-2	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG	Leaf/Root
PER4	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC	Leaf/Root
GADPH	GCTACGCAGAAGACAGTTGATGG	TGGGCACACGGAAGGACATAC	Leaf/Root
EF1a	GGTACCTCCCAGGCTGATTG	TCAGGTAKGAAGACACCTCCTTG	Leaf/Root

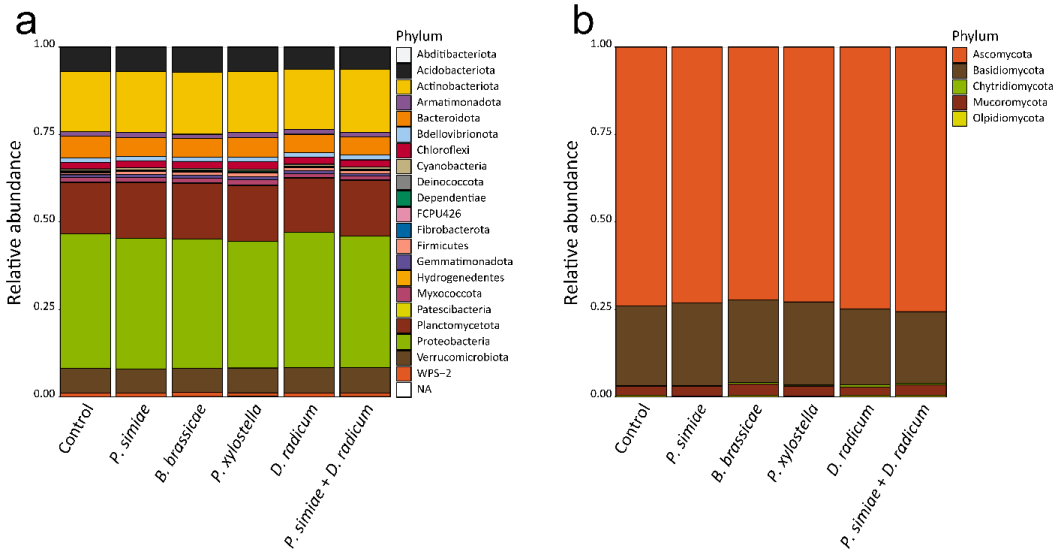


Figure S1 Relative abundance of bacterial (**a**) and fungal (**b**) phyla in rhizospheres of plants exposed to herbivory, rhizobacterial inoculation or a combination.

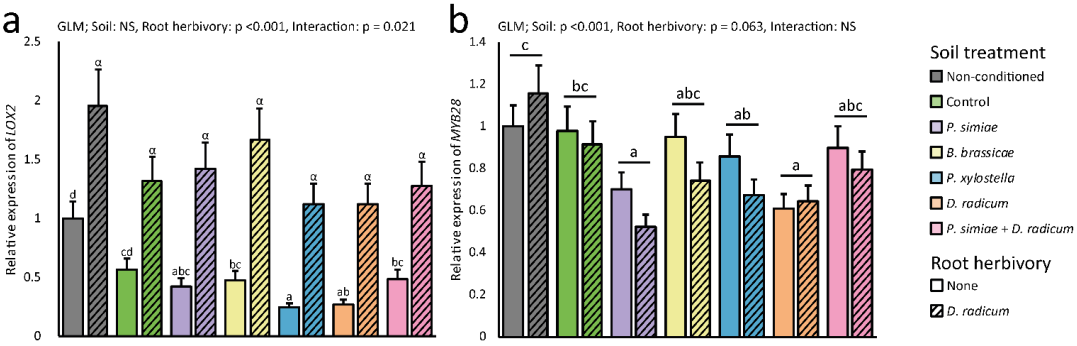


Figure S2 Relative expression of *LOX2* (**a**) and *MYB28* (**b**) in leaves of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417, or infested with *D. radicum* and inoculated with *P. simiae*. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, half of these plants were infested with *D. radicum* (x-axis). All bars are set relative to the gene expression levels in leaves from plants grown in non-conditioned soil and were non-infested (white bar). Bars show mean + SE; different letters are significantly different from one another, within control (Roman alphabet) or *D. radicum* infested (Greek alphabet) samples (Tukey's HSD, α = 0.05). Soil: soil conditioning treatment; *D. radicum: D. radicum* infestation treatment, NS; Not significant, GLM; Generalized Linear Model. N = 3 or 4 replicates of pools of three plants.

Supplemental methods: Delia radicum biomass assessment

To assess performance of *D. radicum* while the larvae are still within the primary root, we developed species-specific primers. As the goal was to be able to measure low quantities of *D. radicum* biomass within cabbage roots, RNA of the 18S ribosomal subunit was targeted. During *in silico* primer development, specificity of *D. radicum* primers was optimized by testing specifically for BLAST hits on Sciaridae, Nematoda, Fungi and *B. oleracea*; as these are hypothesized to be the most common non-target organisms in our samples (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).
One primer pair was further tested (Table **S3**).

Table S3 *Delia radicum* specific primer pairs.

Gene	Forward primer ('5 to 3')	Reverse primer ('3 to 5')	Product size (bp)
18S	GCAAGATCGTTATTATGGTTGAACTCT	GAACCCTGATTCCCCGTTACC	126

Stability of primers across different life stages of *Delia radicum* was confirmed by testing primers on cDNA extracted from neonate larvae, 2- and 3-week-old larvae and pupae (Fig. **S4**). *Delia radicum* RNA was extracted with Isolate II Plant RNA kit (GCbiotech, the Netherlands) following the manufacturer's instructions. RNA was converted to cDNA using cDNA Synthesis Kit (SensiFAST, Bioline). Quantitative polymerase chain reaction (qPCR) analysis was performed to test transcript levels of genes of interest (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). Expression data was processed using qBase and data analyzed in R. Gel electrophoresis and melt curves indicated no non-target products of different lengths. Expression was stable across life-stages for each primer.

To assess the ability of this novel technique to discriminate between different larval densities *in planta*, 3-week-old *B. oleracea* plants were induced with 2, 4, 6, 8, 10 neonate *D. radicum* larvae. After 24 h, primary roots were harvested by uprooting the plants, cutting off secondary roots, and freezing the samples directly in liquid nitrogen. Samples were pooled for three plants. Analysis was performed as described above.

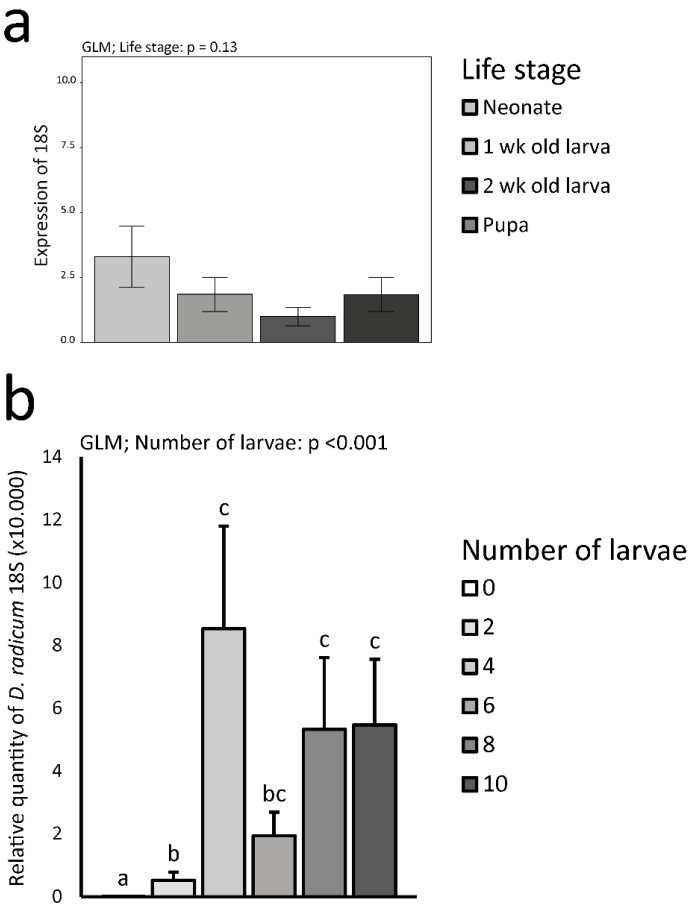


Figure S4 (**a**) Relative gene expression of *D. radicum* 18S in life stages of *D. radicum*; (**b**) the relative quantity of *D. radicum* 18S. Bars show mean ± SE; different letters are significantly different from one another. GLM; Generalized Linear Model. N = 4 insect individuals.

Chapter 7

General discussion

Reprise: The aim of this thesis

A plant's health depends on interactions with an intricate and complex web of macro- and microorganisms living in the soil as well as in or on aboveground tissues (Berendsen *et al.*, 2012; Stam *et al.*, 2014; Rolfe *et al.*, 2019). Certain bacterial species, termed plant growth promoting rhizobacteria (PGPR), that occur in the soil microbial community have shown to promote plant health by stimulating growth and plant defense (van der Heijden *et al.*, 2008). Growth is stimulated by microbial production of plant growth regulators, and/or improvement of plant nutrition by supplying and facilitating nutrient uptake from the soil (Lugtenberg & Kamilova, 2009; Yang *et al.*, 2009; Hayat *et al.*, 2010). PGPR further have been shown to alleviate plant stresses by priming plant defense against an impending attack from pathogens or insect herbivores. This priming allows a plant to respond more quickly and effectively to an actual attack, which then results in a lower performance of the attacker and a better overall performance of the plant (Beneduzi *et al.*, 2012; Pieterse *et al.*, 2014).

Plants respond to herbivory with changes in chemical, physiological, and morphological traits (Ohgushi, 2005; Stam *et al.*, 2014). For plant defense induction, an important factor is attacker identity (Bonaventure, 2012), which governs the specific plant defense and hormonal response. Chewing insects induce defenses that are mainly regulated by the phytohormone jasmonic acid, whereas phloem-feeding insects generally induce defenses that are regulated by the phytohormone salicylic acid (Erb *et al.*, 2012b). The two phytohormones activate intricate signal transduction pathways. These pathways are also instrumental in the rhizobacterial priming of plant defense (Conrath, 2011; Pieterse *et al.*, 2014). Phytohormones and their corresponding pathways are additionally involved in the recruitment of soil bacteria through root exudations (Kim *et al.*, 2016; Sasse *et al.*, 2018). Chapter 2 further focuses on the bi-directional plant-mediated interactions between rhizobacteria and herbivorous insects.

Prior to the studies presented in this thesis, no research had focused on the rhizobacterial effects on the growth and resistance of cabbage plants, *Brassica oleracea*, towards shoot- and root-feeding insects. Therefore, the aim of this thesis was to explore how *B. oleracea* plants respond to the rhizobacterium *Pseudomonas simiae* WCS417r and how these plant responses affect several insect herbivores. Plants were grown in a greenhouse to investigate how different delivery methods of rhizobacteria affect plant defense such as addition of rhizobacteria to the soil (chapter 3) or addition of the rhizobacteria to seeds before sowing (chapter 4). Specifically, in chapter 3 I investigated the effects of *P.*

simiae mixed into the soil on *B. oleracea* plant growth and defense against the root chewer *Delia radicum* and the leaf feeders *Plutella xylostella* and *Mamestra brassicae*. In chapter 4 I examined the effects on plant growth and resistance by adding the rhizobacteria to the seeds instead of the soil. Although seed bacterization has been shown to promote plant growth in oilseed rape and maize (Abuamsha *et al.*, 2011a; O'Callaghan, 2016; Singh *et al.*, 2020), I did not find increased growth of cabbage plants grown from bacterized seeds, compared to plants grown from sterilized seeds. The seed bio-priming technique still is an interesting technique due to being scalable and accessible to farmers. It could be that the protocol used in chapter 4 needs optimization to result in plant growth promotion. Rather than to further work on the development of seed bacterization optimization, I focused on the insect performance results from chapter 3 in chapter 5, in which I reported on plant responses to dual herbivore attack and rhizobacterial soil inoculation, using *D. radicum* and *P. xylostella* as plant attackers. In chapter 6 I went in-depth to focus on *B. oleracea* rhizosphere microbiome changes after shoot- and root herbivory, and explored how rhizobacteria added to the soil as well as insect herbivory affected the plant's rhizosphere microbiome composition. These microbiome compositional changes were then further assessed through assessing growth and defense in a second set of *B. oleracea* plants.

In the present chapter I aim to connect the results of the individual chapters, and discuss important aspects of this thesis and rhizobacteria-plant research.

Rhizobacteria: from laboratory studies to field application

Plant growth medium – an important factor

In plant-bacteria research the choice of plant growth medium determines the nature of the research. Mechanistic mode-of-action research commonly studies the model plant *Arabidopsis thaliana* on nutrient agar (for example (Allard-Massicotte *et al.*, 2016; Pangesti *et al.*, 2016; Asari *et al.*, 2017; Pangesti *et al.*, 2017; Witzel *et al.*, 2017; Stringlis *et al.*, 2018)). Alternatively, plants may be grown gnotobiotically in a nutrient solution in wells (Haney *et al.*, 2015; Stringlis *et al.*, 2018). In agar plates, the exposed root tips are inoculated with a purified bacterial culture in a gnotobiotic environment, which allows confirmation of proper biofilm formation and subsequent colonization compared to roots in soil. These experiments often show a reproducibility that is relatively high compared to experiments involving soil, due to lack of microbial competition and other variable soil factors. However, responses of plants grown on agar may not be comparable to those of plants grown

in soil. Rhizobacterially induced plant growth promotion differs between *A. thaliana* plants grown on plates compared to plants grown in soil (Ryu *et al.*, 2005). Hence, agar as a plant growth medium is unsuitable to simulate plant growth and rhizobacterial promotion in soil.

Sterilized soil might offer a suitable in-between, separating agar and soil as plant growth medium in terms of complexity of biotic and abiotic factors in rhizobacteria-plant experiments. Soil sterilization possibly may aid in rhizobacterial establishment, providing a non-competitive zone for the bacteria to inhabit (van Veen *et al.*, 1997; Li *et al.*, 2019). In non-sterilized soil, the numbers of rhizobacteria have been observed to diminish quickly (van Veen *et al.*, 1997). However, the assumption that rhizobacterial colonization is successful to a higher degree in sterilized soil, thereby providing increased plant growth promotion, is yet to be proven.

Soil sterilization itself can influence plant growth (Mahmood *et al.*, 2014), and further interact with rhizobacterial plant growth promotion. Root respiration, an indicator of root growth, increased in *Cerasus sachalinensis* after addition of the rhizobacterium *Staphylococcus sciuri* to the soil (Zhou *et al.*, 2015). Sterilization in itself did not affect plant growth in this plant species. Yet, the use of sterilized soil canceled out the promotional effects of PGPR addition on root respiration rate (Zhou *et al.*, 2015). Moreira and colleagues (2019) used zinc-contaminated soil to examine the effect of rhizobacterial addition to sterilized and non-sterilized soil with either *Ralstonia eutropha* 1C2 or *Chryseobacterium humi* ECP37. The use of sterilized soil increased maize shoot biomass and decreased root biomass compared to plants in non-sterilized soil (Moreira *et al.*, 2019). Rhizobacterial inoculation in non-sterile soil increased shoot but not root biomass, whereas rhizobacterial inoculation in sterile soil increased both root and shoot biomass (Moreira *et al.*, 2019). These results are interpreted as soil sterilization differently affecting the effect of rhizobacterial inoculation on plant growth. Further, soil sterilization techniques may also differ in their effects on soil nutritional status (Berns *et al.*, 2008). Hence, employment of sterilized soil in the experiments of this thesis may have influenced the rhizobacterially induced plant growth promotion. Sterilized soil is commonly used in experiments investigating the effects of rhizobacterial addition to plants, but rarely examined or discussed as an influential soil factor.

Unpredictability of rhizobacterial addition to soil in field applications

Soil is a highly heterogeneous material that contains a plethora of bacterial species. The many bacterial species can interact with soil biotic and abiotic factors, such as soil pH, soil water content, or its macrofauna.

These soil factors affect a wide range of plant and bacterial responses, which contributes to variable application success to promote growth and/or defense through rhizobacterial priming. One additional factor that contributes to context-dependent responses is the species' genetic make-up, both of the plant as well as the rhizobacteria.

Genetic identity can determine the compatibility between plant species and rhizobacterial strain. For example, two different PGPR strains, *P. fluorescens* GM30 and *P. fluorescens* Pf-5, can provide strain-specific responses in sugar- and nutrient-signaling, calcium signaling, and auxin metabolism of *A. thaliana* (Weston *et al.*, 2012). The same rhizobacterial strain can differently affect several plant species. The PGPR strain *P. fluorescens* LBUM677 differently affected soybean, canola and corn gromwell (*Buglossoides arvensis*) in terms of biomass and seed production (Jimenez *et al.*, 2020). Genetic variation within a plant species also affects rhizobacterial colonization. In *A. thaliana* the same bacterial strain can have different degrees of effectiveness depending on the accession tested for growth promotion (Wintermans *et al.*, 2016). This diversification in plant colonization capacity can lead to variation in plant resistance towards pathogens (Haney *et al.*, 2015). Plant genetic variation can also be reflected in diversification in plant defense responses (Gols *et al.*, 2008), which can further increase the variability between plant-microbe-insect interactions. How genetic variation of each participant interacts with soil factors and herbivory will be engaging questions to further unravel.

For successful field application of PGPR to promote plant growth, not only must there be genetic compatibility, but also sufficient colonization in the rhizosphere community and biofilm formation on the plant itself. The structure of the native soil microbial community may be essential for rhizospheric acceptance of inoculum. The native community residents may outcompete the inoculum microbe species (Hibbing *et al.*, 2010), leading to a rapid decline of inoculum species (van Veen *et al.*, 1997; Compant *et al.*, 2010). However, if the inoculum species is already present in the soil, there may be an established ecological niche for the inoculum to expand its population size. Non-native bacteria such as an inoculum, may be more likely to disrupt the present community, and cause antagonistic effects on the present community resulting in decreased plant growth (Gadhave *et al.*, 2016b). The particular rhizospheric rejection or acceptance of inoculum may be seen in assessments of plant biomass, namely larger size variation. Calabrese *B. oleracea* var. *italica* plants varied more in size after rhizobacterial addition of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. cereus* compared to non-inoculated plants (Gange & Gadhave, 2018). As the rhizosphere is

considered one of the most dynamic places on earth (Philippot *et al.*, 2013), communities of microbial species as well as their competition and/or cooperation in the rhizosphere are hugely complex. Teasing apart the microbe's roles in the community could be achieved with the help of rhizobacterial ecology modelling (Strigul & Kravchenko, 2006; Muci *et al.*, 2012), but the task is monumental and will take some time to be fully mapped and understood.

In the rhizosphere, the relative bacterial abundance may play a role in activation of plant promotion and defense. PGPR *B. pumilus* WP8 population declined after soil inoculation, but still promoted fava bean *Vicia faba* growth (Kang *et al.*, 2013). Instead of directly promoting plant growth, the inoculation affected the soil bacterial community structure which in turn might have contributed to growth promotion (Kang *et al.*, 2013). The role of strain-specific bacterial relative abundance as a main driver for plant growth promotion may be limited. Likely, relative abundance driving plant colonization and subsequent growth promotion would result in more successful field inoculum applications.

Interestingly, it may be that rhizobacterial promotion of plant growth is most effective in stressed soils, or soil with low organic matter content (Martinez-Viveros *et al.*, 2010). Indeed, studies conclude a greater advantage of plant growth promoting effects during plant abiotic stresses (Rubin *et al.*, 2017; Schutz *et al.*, 2018). If the same patterns can be found for biotic stresses is yet to be discovered. It is possible that plants maintain a rich microbial diversity around their roots to be able to recruit specific microbes to aid in stressed situations. For the plant, this would involve balancing fitness costs between possible benefits gained through microbial cooperation, and fitness costs of maintaining the diversity, as well as with possible fitness costs from defending against detrimental microbes (Gadhave *et al.*, 2016b).

Confirmation of strain inoculum in plant rhizosphere bacterial communities

Plants alter their root exudations which in turn affects their rhizosphere microbiome. How the plant root-associated rhizosphere microbiome is affected by herbivore attack was examined in chapter 6. Specifically, this chapter examined how rhizobacterial addition and/or herbivore attack alter plant microbe community. The analysis from unique Amplicon Sequence Variants (ASV´s) differed between plants attacked by shoot- or root-feeding insect herbivores. The manipulation of the rhizosphere microbiome with added rhizobacteria was not detectable in the rhizosphere analysis. Likely, inoculum addition altered a few important soil species through competition with native species. The

conclusions from chapter 6 were that insect attack leads to larger rhizosphere microbiome changes than rhizobacterial addition alone. These results can be interpreted as an inability to detect small differences possibly resulting from rhizobacterial addition in the overall rhizosphere microbiome community in the field, where the plant is exposed to multiple stresses such as insect herbivory, or an indication of strong competition between the inoculum and the resident microbiota.

Rhizobacterial studies focusing on plant performance or insect performance, often exclude a rhizospheric bacterial community assessment (Martinez-Viveros *et al.*, 2010; Rilling *et al.*, 2019). In my thesis I present an overview which shows the low number of studies that include bacterial community assessment compared to the number of studies without bacterial community assessment, in the context of rhizobacterial modifications of plant-insect interactions. New interesting tools for microbial community tracking in soil and solutions have been developed (Krzyzanowska *et al.*, 2012; Massalha *et al.*, 2017), but find limited application so far.

A recent interesting paper suggests biases from sampling of soil microbial community assessments (Tkacz *et al.*, 2020). The authors suggest that differences found in the literature between rhizosphere microbiomes is more dependent on sampling procedures, rather than large actual differences between plant species microbiomes. Due to root architecture differences dependent on e.g. soil water and nutrient availability, different amounts of soil or microbial biofilm stick to the roots. As the microbial difference is greater depending on the distance from the root surface, sampling too much of bulk soil may therefore skew results of soil microbiome analyses. These fascinating new notions still need to be confirmed through additional experiments.

Plant-microbe communication — *MYB72* gene expression

MYB72 is an interesting gene in plant-rhizobacterial studies, as it has been identified in *A. thaliana* to be a node in plant-rhizobacterial communication (Segarra *et al.*, 2009; Pieterse *et al.*, 2014; Romera *et al.*, 2019). However, in other plants it has not been investigated whether *MYB72* fulfils a similar function in plant-rhizobacteria communication. In both *A. thaliana* and tomato, *MYB72* is active in the plant response to iron deficiency (Palmer *et al.*, 2013; Asins *et al.*, 2020). Whether there is a link between iron deficiency response and *MYB72*, and plant-rhizobacteria communication in other plants is unknown. In chapter 6, I targeted the homologue of the *A. thaliana MYB72* gene in *B. oleracea*, with the aim to confirm a role in rhizobacterial colonization similarly to the *A. thaliana*-rhizobacteria system. In this chapter, plants were subjected to different

treatments, including *P. simiae* inoculation. After two weeks, plants were removed, and a set of new plants were grown in the same soil. The second set of plants were sampled for gene expression after three weeks. The results showed no correlation between upregulation of *MYB72* expression and *P. simiae* inoculation of the first set of plants. On the other hand, if *MYB72* upregulation would have been found, this would not unequivocally link this gene to *P. simiae* WCS417r inoculation, as other soil bacteria could be the cause. In addition to the measurements in chapter 6, I examined *B. oleracea* seedlings grown in agar plates with root tip rhizobacterial inoculation, to test whether induction of *MYB72* was upregulated with inoculation compared to expression in non-inoculated plants (unpublished). I found no *MYB72* upregulation after *P. simiae* inoculation. It may be that the *MYB72* homologue has other functions in cabbage than in *A. thaliana*. Alternatively, rhizobacteria-plant communication uses a different pathway in cabbage. Altogether, a different marker gene for rhizobacterial colonization is needed for cabbage. It will be interesting to see whether plants other than *A. thaliana* use a homologue of this gene, or if alternative communication pathways operate in plant-rhizobacteria communication.

Rhizobacterial interactions with insect herbivores mediated by the plant

In this thesis I investigated the insect *D. radicum* and assessed its performance on *B. oleracea* plants. In chapter 3, I surprisingly recorded enhanced performance of *D. radicum* when the insects fed on plants grown on inoculated soil compared to performance of insects that feed on plants grown on mock-inoculated soil. In chapter 4, the performance of *D. radicum* was in one experiment increased, whereas the other experiment showed similar fly weight in response to feeding on plants grown from rhizobacterially bacterized or water-soaked seeds. In chapter 6, *D. radicum* emergence was increased when feeding on plants that grew in soil previously conditioned by plants attacked by *D. radicum* and inoculated with *P. simiae*, compared to plants that grew in soil previously conditioned by plants attacked only by *D. radicum*. In unpublished experiments, I examined *D. radicum* preference between pieces of turnip dipped in rhizobacterial solution and water-dipped pieces of turnip. The neonate larvae preferred rhizobacteria-dipped turnips over water-dipped turnips after 24 h. Together, these results can be understood as representing a benefit for *D. radicum* performance from *P. simiae* soil inoculation.

Previously only a few studies have been performed which include both rhizobacteria and root herbivores. Rhizobacteria negatively affected

the South American corn rootworm *Diabrotica speciosa* performance when feeding on plants inoculated with the rhizobacterium *Azospirillum brasilense* compared to non-inoculated plants (Santos *et al.*, 2014). The contrast between the latter study and the results presented in this thesis, suggests differences between insect species, plant species and/or rhizobacterial strain. In addition, the plant genotype can influence *D. radicum* performance. Insect survival was altered depending on the cabbage population studied (van Geem *et al.*, 2015). It would be interesting to learn whether *D. radicum* can benefit from addition of other rhizobacterial strains as well, and how these possible benefits affect interactions with plant genotypes.

Two other insects included in this thesis were *P. xylostella* and *M. brassicae*. The performance of *P. xylostella* and *M. brassicae* was examined in chapters 3 and 4, and *P. xylostella* performance in the dual herbivore experiment included in chapter 5. The results for *P. xylostella* varied. On the one hand, the results in chapter 3 show a negative effect on *P. xylostella* when adding *P. simiae* to soil. On the other hand, I found no negative effect from *P. simiae* soil inoculation on *P. xylostella* in chapter 5, where the insect weight was similar when feeding on inoculated or non-inoculated plants. These results suggest a stronger context dependency for the performance of *P. xylostella* compared to the performance of *D. radicum*. Perhaps there are unknown factors influencing *P. xylostella* performance that have a stronger effect than rhizobacteria-inoculated soil.

Regarding the performance of *M. brassicae*, results in both chapters 3 and 5 show that larval performance was not affected by adding *P. simiae* to the soil. Previous research on *A. thaliana* plants with this species showed that *M. brassicae* larval performance depends on soil composition ranging from decreased performance to increased performance, compared to control herbivore performance (Pangesti *et al.*, 2015a). Hence, not only soil composition, but also plant species contributes to *M. brassicae* performance when feeding on plants inoculated with *P. simiae*.

Other parameters than insect performance could be affected by feeding on or sensing a rhizobacterially inoculated plant. These parameters could include oviposition choice, host plant choice, or ingested amount of plant tissue. Chapter 5 in this thesis showed rhizobacterial inoculation to reduce the amount of consumed leaf area by feeding *P. xylostella* compared to leaf area consumed by insects feeding on non-inoculated plants. Whether the decrease in consumed leaf area was caused by higher nutritional quality of the leaves is unknown. Previous literature shows that rhizobacteria can alter herbivore insect choice for its

host plant (Zehnder *et al.*, 1997; Santos *et al.*, 2014; Grunseich *et al.*, 2020). In addition, interesting field studies show that the insect community changes after rhizobacterial addition to rice grown in field plots (Commare *et al.*, 2002), and *B. oleracea* grown in fields (Gadhave *et al.*, 2016a). In this thesis the insects were restricted to a single plant, providing no further insight into their host-plant choice. Nonetheless, the insect could move freely on the plant, to encourage a more natural feeding behavior compared to other commonly used research set-up methods such as clip-cages or detached leaves.

In conclusion, in this thesis I found varied effects of rhizobacterial addition on insect performance *via* plant mediated effects. Insect performance depended on the rhizobacterial delivery method, and the biotic and abiotic history of the soil.

Thesis limitations

This thesis focused on biomass of shoots, which limits the knowledge of rhizobacterially induced biomass changes to the shoot. In additional studies on seedlings grown in agar plates, I found that *B. oleracea* seedlings have larger root biomass after rhizobacterial root tip inoculation compared to root biomass from mock-inoculated seedlings (unpublished). In soil, cabbage roots are thin and difficult to harvest in intact state. Future experiments could grow *B. oleracea* in different potting materials, e.g. roots growing in sand may be easier to extract. However, these materials may differ in water content or oxygen availability, possibly modifying native microbial community and subsequent inoculum colonization success.

Confirmation of the presence and abundance of the inoculum strain was included as a result in chapter 3, through selective plate colony count. Microbial measurements and colonization confirmation was uninformative due to not yet identified factors in chapters 4 and 5, and therefore not disclosed within this thesis.

Conclusions and future perspectives

Plants interact with macro- and microorganisms, some organisms being detrimental, others beneficial to plant health (Berendsen *et al.*, 2012; Stam *et al.*, 2014; Rolfe *et al.*, 2019). Some beneficial microbes have potential to increase plant growth and defense of their host plant (Mhlongo *et al.*, 2020). Rhizobacteria have been shown to influence plant defense against insect herbivores *via* plant mediated effects (Pangesti *et al.*, 2015b). Rhizobacterially induced growth promotion and plant defense are interesting features to apply to agricultural crop plants. To employ microbes in agriculture, the microbes need to have predictable effects on

the host plant's performance. The context dependencies between plant, microbe and insect, which appear from the present study and the literature may pose difficulties to application in agriculture.

Microbial application in agriculture requires understanding of soil and plant factors. Colonization success depends on biotic and abiotic soil factors affecting inoculum establishment. Genetic variation within plant species and rhizobacteria may further increase variation in colonization success. In turn, these variations in colonization success can lead to discrepancies in rhizobacteria- induced plant defense. I see several areas of special interest that may advance this field or research:

Is there a threshold bacterial concentration to induce systemic resistance against insects? A bacterial concentration threshold is known for induced systemic resistance against *Fusarium oxysporum* in radish (*Raphanus sativus*) inoculated with the strains *Pseudomonas putida* WCS385 and *P. fluorescence* WCS374 (Raaijmakers *et al.*, 1995). However, how other plant species, bacterial strains and soils affect this threshold value is unknown. In addition, this study involves a pathogen, whereas bacterial threshold values for insect herbivore-induced resistance are not known. Microbial soil communities can also allow a general protection against soil-borne pathogens, this phenomenon is called disease suppressive soil (Exposito *et al.*, 2017). Can microbial soil communities collectively also feature a general insect herbivory suppressiveness, *i.e.* is there an "insect suppressive soil"? (Hokkanen & Menzler-Hokkanen, 2018).

How are rhizobacteria-plant-insect interactions affected by fertilizers? Plants under higher soil nutrient stress may be more likely to associate with beneficial microbes. May then the usage of beneficial microbes be combined with fertilizers in industrialized agriculture? Some studies suggest a combination is possible to reduce fertilizer amount (Adesemoye *et al.*, 2009; Sripontan *et al.*, 2014), but to what extent is still unclear. However, insects are generally benefited by fertilizers (Butler *et al.*, 2012). Does a combination of fertilizers and beneficial microbes affect insect performance?

These questions build on the results and outcomes of this thesis. The results presented demonstrate that the status of the soil influences plant-rhizobacterial interactions (sterilized or non-sterilized), together with the delivery method of the bacteria (added into soil or on the seed), and the legacy effects from previously grown plants in the same soil are important in the plant-rhizobacteria interaction. Inoculated soil with *P. simiae* in which *B. oleracea* plants grow influenced the performance of *D. radicum* positively. Moreover, *P. simiae* treatment influenced the performance of *P. xylostella* and *M. brassica* negatively, or did not affect the performance of the insects. Such differential treatment effects should be considered when applying a rhizobacterium in pest management.

Acknowledgments

I thank Marcel Dicke and Joop van Loon for their comments on a previous version on this chapter.

References

Abuamsha R, Salman M, Ehlers RU. 2011a. Effect of seed priming with *Serratia plymuthica* and *Pseudomonas chlororaphis* to control *Leptosphaeria maculans* in different oilseed rape cultivars. *European Journal of Plant Pathology* **130**(3): 287-295.

Abuamsha R, Salman M, Ehlers RU. 2011b. Improvement of seed bio-priming of oilseed rape (*Brassica napus* ssp. *oleifera*) with *Serratia plymuthica* and *Pseudomonas chlororaphis*. *Biocontrol Science and Technology* **21**(2): 199-213.

Adesemoye AO, Torbert HA, Kloepper JW. 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial Ecology* **58**(4): 921-929.

Allard-Massicotte R, Tessier L, Lecuyer F, Lakshmanan V, Lucier JF, Garneau D, Caudwell L, Vlamakis H, Bais HP, Beauregard PB. 2016. *Bacillus subtilis* early colonization of *Arabidopsis thaliana* roots involves multiple chemotaxis receptors. *mBio* **7**(6): e01664.

Amprayn KO, Rose MT, Kecskes M, Pereg L, Nguyen HT, Kennedy IR. 2012. Plant growth promoting characteristics of soil yeast (*Candida tropicalis* HY) and its effectiveness for promoting rice growth. *Applied Soil Ecology* **61**: 295-299.

Ando Y, Utsumi S, Ohgushi T. 2017. Aphid as a network creator for the plant-associated arthropod community and its consequence for plant reproductive success. *Functional Ecology* **31**(3): 632-641.

Asari S, Tarkowska D, Rolcik J, Novak O, Palmero DV, Bejai S, Meijer J. 2017. Analysis of plant growth-promoting properties of *Bacillus amyloliquefaciens* UCMB5113 using *Arabidopsis thaliana* as host plant. *Planta* **245**(1): 15-30.

Asins MJ, Raga MV, Torrent D, Roca D, Carbonell EA. 2020. QTL and candidate gene analyses of rootstock-mediated tomato fruit yield and quality traits under low iron stress. *Euphytica* **216**(4): 63.

Aznar A, Dellagi A. 2015. New insights into the role of siderophores as triggers of plant immunity: what can we learn from animals? *Journal of Experimental Botany* **66**(11): 3001-3010.

Bacilio-Jimenez M, Aguilar-Flores S, Ventura-Zapata E, Perez-Campos E, Bouquelet S, Zenteno E. 2003. Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. *Plant and Soil* **249**(2): 271-277.

Badri DV, Vivanco JM. 2009. Regulation and function of root exudates. *Plant, Cell and Environment* **32**(6): 666-681.

Badri DV, Zolla G, Bakker MG, Manter DK, Vivanco JM. 2013. Potential impact of soil microbiomes on the leaf metabolome and on herbivore feeding behavior. *New Phytologist* **198**(1): 264-273.

Bakker P, Ran LX, Mercado-Blanco J. 2014. Rhizobacterial salicylate production provokes headaches! *Plant and Soil* **382**(1-2): 1-16.

Ballhorn DJ, Kautz S, Schadler M. 2013. Induced plant defense via volatile production is dependent on rhizobial symbiosis. *Oecologia* **172**(3): 833-846.

Balmer A, Pastor V, Gamir J, Flors V, Mauch-Mani B. 2015. The 'prime-ome': towards a holistic approach to priming. *Trends in Plant Science* **20**(7): 443-452.

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez JP. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). *Plant and Soil* **378**(1-2): 1-33.

Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**(1): 1-48.

Beauregard PB, Chai YR, Vlamakis H, Losick R, Kolter R. 2013. *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proceedings of the National Academy of Sciences of the United States of America* **110**(17): E1621-E1630.

Beneduzi A, Ambrosini A, Passaglia LMP. 2012. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology* **35**(4): 1044-1051.

Bennett JA, Klironomos J. 2019. Mechanisms of plant-soil feedback: interactions among biotic and abiotic drivers. *New Phytologist* **222**(1): 91-96.

Berendsen RL, Pieterse CMJ, Bakker P. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* **17**(8): 478-486.

Berendsen RL, van Verk MC, Stringlis IA, Zamioudis C, Tommassen J, Pieterse CMJ, Bakker P. 2015. Unearthing the genomes of plant-beneficial *Pseudomonas* model strains WCS358, WCS374 and WCS417. *BMC Genomics* **16**: 539

Berendsen RL, Vismans G, Yu K, Song Y, de Jonge R, Burgman WP, Burmolle M, Herschend J, Bakker P, Pieterse CMJ. 2018. Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME Journal* **12**(6): 1496-1507.

Berg G, Raaijmakers JM. 2018. Saving seed microbiomes. *ISME Journal* **12**(5): 1167-1170.

Berg G, Smalla K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology* **68**(1): 1-13.

Berns AE, Philipp H, Narres HD, Burauel P, Vereecken H, Tappe W. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. *European Journal of Soil Science* **59**(3): 540-550.

Bezemer TM, van Dam NM. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology & Evolution* **20**(11): 617-624.

Bezemer TM, van der Putten WH, Martens H, van de Voorde TFJ, Mulder PPJ, Kostenko O. 2013. Above- and below-ground herbivory effects on below-ground plant–fungus interactions and plant–soil feedback responses. *Journal of Ecology* **101**(2): 325-333.

Biere A, Goverse A. 2016. Plant-mediated systemic interactions between pathogens, parasitic nematodes, and herbivores above- and belowground. *Annual Review of Phytopathology* **54**: 499-527.

Blubaugh CK, Carpenter-Boggs L, Reganold JP, Schaeffer RN, Snyder WE. 2018. Bacteria and competing herbivores weaken top-down and bottom-up aphid suppression. *Frontiers in Plant Science* **9**: 1239.

Bonanomi G, Sicurezza MG, Caporaso S, Esposito A, Mazzoleni S. 2006. Phytotoxicity dynamics of decaying plant materials. *New Phytologist* **169**(3): 571-578.

Bonaventure G. 2012. Perception of insect feeding by plants. *Plant Biology* **14**(6): 872-880.

Boutard-Hunt C, Smart CD, Thaler J, Nault BA. 2009. Impact of plant growth-promoting rhizobacteria and natural enemies on *Myzus persicae* (Hemiptera: Aphididae) infestations in pepper. *Journal of Economic Entomology* **102**(6): 2183-2191.

Bulgarelli D, Schlaeppi K, Spaepen S, van Themaat EVL, Schulze-Lefert P. 2013. Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology* **64**: 807-838.

Butler J, Garratt MPD, Leather SR. 2012. Fertilisers and insect herbivores: a meta-analysis. *Annals of Applied Biology* **161**(3): 223-233.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**(7): 581-587.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 4516-4522.

Carrion VJ, Perez-Jaramillo J, Cordovez V, Tracanna V, de Hollander M, Ruiz-Buck D, Mendes LW, van Ijcken WFJ, Gomez-Exposito R, Elsayed SS, et al. 2019. Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* **366**(6465): 606-612.

Carvalhais LC, Dennis PG, Badri DV, Kidd BN, Vivanco JM, Schenk PM. 2015. Linking jasmonic acid signaling, root exudates, and rhizosphere microbiomes. *Molecular Plant-Microbe Interactions* **28**(9): 1049-1058.

Carvalhais LC, Dennis PG, Schenk PM. 2014. Plant defence inducers rapidly influence the diversity of bacterial communities in a potting mix. *Applied Soil Ecology* **84**: 1-5.

Chale-Carrillo VM, Ruiz-Sanchez E, Reyes-Ramirez A, Borges-Gomez L, Cristobal-Alejo J, Pacheco-Aguirre J. 2016. Growth and response to *Bemisia tabaci* in different *Capsicum annuum* genotypes inoculated with *Brevibacillus brevis* CBTC1. *Agrociencia* **50**(3): 323-334.

Chamam A, Sanguin H, Bellvert F, Meiffren G, Comte G, Wisniewski-Dye F, Bertrand C, Prigent-Combaret C. 2013. Plant secondary metabolite profiling evidences strain-dependent effect in the *Azospirillum-Oryza* sativa association. *Phytochemistry* **87**: 65-77.

Ciccillo F, Fiore A, Bevivino A, Dalmastri C, Tabacchioni S, Chiarini L. 2002. Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizospheric bacterial diversity. *Environmental Microbiology* **4**(4): 238-245.

Commare RR, Nandakumar R, Kandan A, Suresh S, Bharathi M, Raguchander T, Samiyappan R. 2002. *Pseudomonas fluorescens* based bio-formulation for the management of sheath blight disease and leaffolder insect in rice. *Crop Protection* **21**(8): 671-677.

Compant S, Clement C, 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 & Biochemistry* **42**(5): 669-678.

Conrath U. 2011. Molecular aspects of defence priming. *Trends in Plant Science* **16**(10): 524-531.

Conrath U, Beckers GJM, Flors V, Garcia-Agustin P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ, et al. 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* **19**(10): 1062-1071.

Cordovez V, Dini-Andreote F, Carrion VJ, Raaijmakers JM. 2019. Ecology and evolution of plant microbiomes. *Annual Review of Microbiology* **73**: 69-88.

Crialesi PCB, Thuler RT, Iost FH, Thuler AMG, Lemos MVF, De Bortoli SA. 2017. Plant growth promoting rhizobacteria (PGPR) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) interaction as a resistance inductor factor in *Brassica oleracea* var. *capitata*. *Plant Science Today* **4**(3): 121-132.

D'Alessandro M, Erb M, Ton J, Brandenburg A, Karlen D, Zopfi J, Turlings TCJ. 2014. Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. *Plant, Cell and Environment* **37**(4): 813-826.

Dawson LA, Grayston SJ, Murray PJ, Ross JM, Reid EJ, Treonis AM. 2004. Impact of *Tipula paludosa* larvae on plant growth and the soil microbial community. *Applied Soil Ecology* **25**(1): 51-61.

De-La-Pena C, Lei Z, Watson BS, Sumner LW, Vivanco JM. 2008. Root-microbe communication through protein secretion. *Journal of Biological Chemistry* **283**(37): 25247-25255.

De Long JR, Fry EL, Veen GF, Kardol P. 2019. Why are plant-soil feedbacks so unpredictable, and what to do about it? *Functional Ecology* **33**(1): 118-128.

de Souza JT, Arnould C, Deulvot C, Lemanceau P, Gianinazzi-Pearson V, Raaijmakers JM. 2003. Effect of 2,4-diacetylphloroglucinol on pythium: cellular responses and variation in sensitivity among propagules and species. *Phytopathology* **93**(8): 966-975.

Dean JM, Mescher MC, De Moraes CM. 2009. Plant-rhizobia mutualism influences aphid abundance on soybean. *Plant and Soil* **323**(1-2): 187-196.

Dean JM, Mescher MC, De Moraes CM. 2014. Plant dependence on rhizobia for nitrogen influences induced plant defenses and herbivore performance. *International Journal of Molecular Sciences* **15**(1): 1466-1480.

Delignette-Muller M, Dutang C. 2015. Fitdistrplus: an R package for fitting distributions. *Journal of Statistical Software* **64**(4): 1-34.

Delory BM, Schempp H, Spachmann SM, Störzer L, van Dam NM, Temperton VM, Weinhold A. 2020. The rhizosphere metabolome triggers species-specific and context-dependent root responses in later arriving plants. *Plant, Cell & Environment* **44**(4): 1215-1230.

Dematheis F, Zimmerling U, Flocco C, Kurtz B, Vidal S, Kropf S, Smalla K. 2012. Multitrophic interaction in the rhizosphere of maize: root feeding of western corn rootworm larvae alters the microbial community composition. *PLoS ONE* **7**(5): e37288.

Dennis PG, Miller AJ, Hirsch PR. 2010. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiology Ecology* **72**(3): 313-327.

Deutsch CA, Tewksbury JJ, Tigchelaar M, Battisti DS, Merrill SC, Huey RB, Naylor RL. 2018. Increase in crop losses to insect pests in a warming climate. *Science* **361**(6405): 916-919.

Dicke M, Baldwin IT. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science* **15**(3): 167-175.

Dini-Andreote F, Raaijmakers JM. 2018. Embracing community ecology in plant microbiome research. *Trends in Plant Science* **23**(6): 467-469.

Disi JO, Zebelo S, Kloepper JW, Fadamiro H. 2018. Seed inoculation with beneficial rhizobacteria affects European corn borer (Lepidoptera: Pyralidae) oviposition on maize plants. *Entomological Science* **21**(1): 48-58.

Doornbos RF, van Loon LC, Bakker P. 2009. Beneficial *Pseudomonas* spp. have altered root colonization on *Arabidopsis thaliana* mutants affected in the expression of induced systemic resistance. *IOBC/wprs Bulletin* **43**: 307-310.

Drogue B, Doré H, Borland S, Wisniewski-Dyé F, Prigent-Combaret C. 2012. Which specificity in cooperation between phytostimulating rhizobacteria and plants? *Research in Microbiology* **163**(8): 500-510.

EIP-AGRI. 2016. Focus Group on IPM: IPM for Brassica. Belgium: The European Innovation Partnership Agricultural Productivity and Sustainability.

Erb M, Flors V, Karlen D, de Lange E, Planchamp C, D'Alessandro M, Turlings TCJ, Ton J. 2009. Signal signature of aboveground-induced resistance upon belowground herbivory in maize. *Plant Journal* **59**(2): 292-302.

Erb M, Glauser G, Robert CAM. 2012a. Induced immunity against belowground insect herbivores- activation of defenses in the absence of a jasmonate burst. *Journal of Chemical Ecology* **38**(6): 629-640.

Erb M, Meldau S, Howe GA. 2012b. Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science* **17**(5): 250-259.

Exposito RG, de Bruijn I, Postma J, Raaijmakers JM. 2017. Current insights into the role of rhizosphere bacteria in disease suppressive soils. *Frontiers in Microbiology* **8**: 2529.

Fahimi A, Ashouri A, Ahmadzadeh M, Hoseini Naveh V, Asgharzadeh A, Maleki F, Felton GW. 2014. Effect of PGPR on population growth parameters of cotton aphid. *Archives of Phytopathology and Plant Protection* **47**(11): 1274-1285.

Fernández de Bobadilla MF, Friman J, Pangesti N, Dicke M, van Loon JJA, Pineda A. 2017. Does drought stress modify the effects of plant-growth promoting rhizobacteria on an aboveground chewing herbivore? *Insect Science* **24**(6): 1034-1044.

Fox J, Weisberg S 2019. Companion to applied regression, Third Edition: Thousand Oaks CA, Sage.

Friman J, Pineda A, Gershenzon J, Dicke M, van Loon JJA. 2021a. Differential effects of the rhizobacterium *Pseudomonas simiae* on above- and belowground chewing insect herbivores. *Journal of Applied Entomology* **145**(3): 250-260.

Friman J, Pineda A, van Loon JJA, Dicke M. 2021b. Bidirectional plant-mediated interactions between rhizobacteria and shoot-feeding herbivorous insects: a community ecology perspective. *Ecological Entomology* **46**(1): 1–10.

Furlong MJ, Wright DJ, Dosdall LM. 2013. Diamondback moth ecology and management: problems, progress, and prospects. *Annual Review of Entomology* **58**: 517-541.

Gadhave KR, Devlin PF, Ebertz A, Ross A, Gange AC. 2018. Soil inoculation with *Bacillus* spp. modifies root endophytic bacterial diversity, evenness, and community composition in a context-specific manner. *Microbial Ecology* **76**(3): 741-750.

Gadhave KR, Finch P, Gibson TM, Gange AC. 2016a. Plant growth-promoting *Bacillus* suppress *Brevicoryne brassicae* field infestation and trigger density-dependent and density-independent natural enemy responses. *Journal of Pest Science* **89**(4): 985-992.

Gadhave KR, Hourston JE, Gange AC. 2016b. Developing soil microbial inoculants for pest management: can one have too much of a good thing? *Journal of Chemical Ecology* **42**(4): 348-356.

Gange AC, Gadhave KR. 2018. Plant growth-promoting rhizobacteria promote plant size inequality. *Scientific Reports* **8**: 13828.

Gange AC, Smith AK. 2005. Arbuscular mycorrhizal fungi influence visitation rates of pollinating insects. *Ecological Entomology* **30**(5): 600-606.

Getman-Pickering ZL, Campbell A, Aflitto N, Grele A, Davis JK, Ugine TA. 2020. LeafByte: a mobile application that measures leaf area and herbivory quickly and accurately. *Methods in Ecology and Evolution* **11**(2): 215-221.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA. 2008. Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. *Ecology* **89**(6): 1616-1626.

Grunseich JM, Thompson MN, Aguirre NM, Helms AM. 2020. The role of plant-associated microbes in mediating host-plant selection by insect herbivores. *Plants-Basel* **9**(1): 6.

Haney CH, Samuel BS, Bush J, Ausubel FM. 2015. Associations with rhizosphere bacteria can confer an adaptive advantage to plants. *Nature Plants* **1**(6): 15051.

Hassan S, Mathesius U. 2012. The role of flavonoids in root–rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions. *Journal of Experimental Botany* **63**(9): 3429-3444.

Hayat R, Ali S, Amara U, Khalid R, Ahmed I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology* **60**(4): 579-598.

Hein JW, Wolfe GV, Blee KA. 2008. Comparison of rhizosphere bacterial communities in *Arabidopsis thaliana* mutants for systemic acquired resistance. *Microbial Ecology* **55**(2): 333-343.

Herman MAB, Nault BA, Smart CD. 2008. Effects of plant growth-promoting rhizobacteria on bell pepper production and green peach aphid infestations in New York. *Crop Protection* **27**(6): 996-1002.

Herschkovitz Y, Lerner A, Davidov Y, Rothballer M, Hartmann A, Okon Y, Jurkevitch E. 2005. Inoculation with the plant-growth-promoting rhizobacterium *Azospirillum brasilense* causes little disturbance in the rhizosphere and rhizoplane of maize (*Zea mays*). *Microbial Ecology* **50**(2): 277-288.

Hervé M. 2020. Package 'RVAideMemoire'. *See https://CRAN. R-project. org/package= RVAideMemoire*: Version 0.9-52. The Comprehensive R Archive Network, Vienna, Austria, 2015.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB. 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology* **8**(1): 15-25.

Hilker M, Schwachtje J, Baier M, Balazadeh S, Baurle I, Geiselhardt S, Hincha DK, Kunze R, Mueller-Roeber B, Rillig MG, et al. 2016. Priming and memory of stress responses in organisms lacking a nervous system. *Biological Reviews* **91**(4): 1118-1133.

Hokkanen HMT, Menzler-Hokkanen I. 2018. Insect pest suppressive soils: buffering pulse cropping systems against outbreaks of *Sitona* weevils. *Annals of the Entomological Society of America* **111**(4): 139-143.

Hol WHG, Bezemer TM, Biere A. 2013. Getting the ecology into interactions between plants and the plant growth-promoting bacterium *Pseudomonas fluorescens*. *Frontiers in Plant Science* **4**: 81.

Holland JN, Cheng WX, Crossley DA. 1996. Herbivore-induced changes in plant carbon allocation: assessment of below-ground C fluxes using carbon-14. *Oecologia* **107**(1): 87-94.

Howe GA, Jander G. 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59**: 41-66.

Hu LF, Robert CAM, Cadot S, Zhang X, Ye M, Li BB, Manzo D, Chervet N, Steinger T, van der Heijden MGA, et al. 2018. Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications* **9**: 2738.

Huang W, Siemann E, Xiao L, Yang XF, Ding JQ. 2014. Species-specific defence responses facilitate conspecifics and inhibit heterospecifics in above-belowground herbivore interactions. *Nature Communications* **5**: 4851.

Huang XF, Chaparro JM, Reardon KF, Zhang RF, Shen QR, Vivanco JM. 2014. Rhizosphere interactions: root exudates, microbes, and microbial communities. *Botany* **92**(4): 267–275.

Ihrmark K, Bodeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandstrom-Durling M, Clemmensen KE, et al. 2012. New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology* **82**(3): 666-677.

Indiragandhi P, Anandham R, Madhaiyan M, Sa TM. 2008. Characterization of plant growth-promoting traits of bacteria isolated from larval guts of diamondback moth *Plutella xylostella* (Lepidoptera : Plutellidae). *Current Microbiology* **56**(4): 327-333.

Jayapala N, Mallikarjunaiah NH, Puttaswamy H, Gavirangappa H, Ramachandrappa NS. 2019. Rhizobacteria *Bacillus* spp. induce resistance against anthracnose disease in chili (*Capsicum annuum* L.) through activating host defense response. *Egyptian Journal of Biological Pest Control* **29**: 45.

Jimenez JA, Novinscak A, Filion M. 2020. *Pseudomonas fluorescens* LBUM677 differentially increases plant biomass, total oil content and lipid composition in three oilseed crops. *Journal of Applied Microbiology* **128**(4): 1119-1127.

Johnson SN, Erb M, Hartley SE. 2016. Roots under attack: contrasting plant responses to below- and aboveground insect herbivory. *New Phytologist* **210**(2): 413-418.

Johnson SN, Mitchell C, McNicol JW, Thompson J, Karley AJ. 2013. Downstairs drivers - root herbivores shape communities of above-ground herbivores and natural enemies via changes in plant nutrients. *Journal of Animal Ecology* **82**(5): 1021-1030.

Kafle D, Hanel A, Lortzing T, Steppuhn A, Wurst S. 2017. Sequential above-and belowground herbivory modifies plant responses depending on herbivore identity. *BMC Ecology* **17**: 5.

Kan JH, Fang RX, Jia YT. 2017. Interkingdom signaling in plant-microbe interactions. *Science China-Life Sciences* **60**(8): 785-796.

Kang YJ, Shen M, Wang HL, Zhao QX. 2013. A possible mechanism of action of plant growth-promoting rhizobacteria (PGPR) strain *Bacillus pumilus* WP8 via regulation of soil bacterial community structure. *Journal of General and Applied Microbiology* **59**(4): 267-277.

Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF. 2008. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* **89**(2): 392-406.

Kariyat RR, Hardison SB, De Moraes CM, Mescher MC. 2017. Plant spines deter herbivory by restricting caterpillar movement. *Biology Letters* **13**(5): 20170176.

Karssemeijer PN, Reichelt M, Gershenzon J, van Loon JJA, Dicke M. 2020. Foliar herbivory by caterpillars and aphids differentially affects phytohormonal signalling in roots and plant defence to a root herbivore. *Plant, Cell & Environment* **43**(3): 775-786.

Kim B, Song GC, Ryu CM. 2016. Root exudation by aphid leaf infestation recruits root-associated *Paenibacillus* spp. to lead plant insect susceptibility. *Journal of Microbiology and Biotechnology* **26**(3): 549-557.

Kim J, Felton GW. 2013. Priming of antiherbivore defensive responses in plants. *Insect Science* **20**(3): 273-285.

Kim YC, Anderson AJ. 2018. Rhizosphere pseudomonads as probiotics improving plant health. *Molecular Plant Pathology* **19**(10): 2349-2359.

Kloepper JW, Ryu CM, Zhang SA. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**(11): 1259-1266.

Kniskern JM, Traw MB, Bergelson J. 2007. Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* **20**(12): 1512-1522.

Kong CH, Zhang SZ, Li YH, Xia ZC, Yang XF, Meiners SJ, Wang P. 2018. Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. *Nature Communications* **9**: 3867.

Kong HG, Kim BK, Song GC, Lee S, Ryu CM. 2016. Aboveground whitefly infestation-mediated reshaping of the root microbiota. *Frontiers in Microbiology* **7**: 1314

Kos M, Tuijl MAB, de Roo J, Mulder PPJ, Bezemer TM. 2015a. Plant-soil feedback effects on plant quality and performance of an aboveground herbivore interact with fertilisation. *Oikos* **124**(5): 658-667.

Kos M, Tuijl MAB, de Roo J, Mulder PPJ, Bezemer TM. 2015b. Species-specific plant-soil feedback effects on above-ground plant-insect interactions. *Journal of Ecology* **103**(4): 904-914.

Kostenko O, Bezemer TM. 2020. Abiotic and biotic soil legacy effects of plant diversity on plant performance. *Frontiers in Ecology and Evolution* **8**: 87.

Kostenko O, van de Voorde TFJ, Mulder PPJ, van der Putten WH, Bezemer TM. 2012. Legacy effects of aboveground-belowground interactions. *Ecology Letters* **15**(8): 813-821.

Krzyzanowska D, Obuchowski M, Bikowski M, Rychlowski M, Jafra S. 2012. Colonization of potato rhizosphere by GFP-tagged *Bacillus subtilis* MB73/2, *Pseudomonas* sp. P482 and *Ochrobactrum* sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. *Sensors* **12**(12): 17608-17619.

Kulmatiski A, Beard KH, Norton JM, Heavilin JE, Forero LE, Grenzer J. 2017. Live long and prosper: plant-soil feedback, lifespan, and landscape abundance covary. *Ecology* **98**(12): 3063-3073.

Lachaise T, Ourry M, Lebreton L, Guillerm-Erckelboudt AY, Linglin J, Paty C, Chaminade V, Marnet N, Aubert J, Poinsot D, et al. 2017. Can soil microbial diversity influence plant metabolites and life history traits of a rhizophagous insect? A demonstration in oilseed rape. *Insect Science* **24**(6): 1045-1056.

Lamers JG, Schippers B, Geels FP. 1988. Soil-borne diseases of wheat in the Netherlands and results of seed bacterization with Pseudomonads against *Gaeumannomyces graminis* var. tritici. *Cereal breeding related to integrated cereal production. Pudoc, Wageningen*: 134–139.

Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, Malfatti S, del Rio TG, Jones CD, Tringe SG, et al. 2015. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* **349**(6250): 860-864.

Lee B, Lee S, Ryu CM. 2012. Foliar aphid feeding recruits rhizosphere bacteria and primes plant immunity against pathogenic and non-pathogenic bacteria in pepper. *Annals of Botany* **110**(2): 281-290.

Lekberg Y, Bever JD, Bunn RA, Callaway RM, Hart MM, Kivlin SN, Klironomos J, Larkin BG, Maron JL, Reinhart KO, et al. 2018. Relative importance of competition and plant-soil feedback, their synergy, context dependency and implications for coexistence. *Ecology Letters* **21**(8): 1268-1281.

Lenth R, Love J, Herve M. 2018. Package emmeans. *See https://CRAN. R-project. org/package= emmeans*: The Comprehensive R Archive Network, Vienna, Austria, 2015.

Li K, DiLegge MJ, Minas IS, Hamm A, Manter D, Vivanco JM. 2019. Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. *Rhizosphere* **12**: 100176

Li YH, Dicke M, Kroes A, Liu W, Gols R. 2016a. Interactive effects of cabbage aphid and caterpillar herbivory on transcription of plant genes associated with phytohormonal signalling in wild cabbage. *Journal of Chemical Ecology* **42**(8): 793-805.

Li YH, Xia ZC, Kong CH. 2016b. Allelobiosis in the interference of allelopathic wheat with weeds. *Pest Management Science* **72**(11): 2146-2153.

Liu HW, Carvalhais LC, Schenk PM, Dennis PG. 2017. Effects of jasmonic acid signalling on the wheat microbiome differ between body sites. *Scientific Reports* **7**: 41766.
Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**(12): 550.
Lu J, Robert CAM, Riemann M, Cosme M, Mene-Saffrane L, Massana J, Stout MJ, Lou YG, Gershenzon J, Erb M. 2015. Induced jasmonate signaling leads to contrasting effects on root damage and herbivore performance. *Plant Physiology* **167**(3): 1100-1116.
Lugtenberg B, Kamilova F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* **63**: 541-556.
Lukwinski AT, Hill JE, Khachatourians GG, Hemmingsen SM, Hegedus DD. 2006. Biochemical and taxonomic characterization of bacteria associated with the crucifer root maggot (*Delia radicum*). *Canadian Journal of Microbiology* **52**(3): 197-208.
Ma HK, Pineda A, van der Wurff AWG, Raaijmakers C, Bezemer TM. 2017. Plant-soil feedback effects on growth, defense and susceptibility to a soil-borne disease in a cut flower crop: species and functional group effects. *Frontiers in Plant Science* **8**: 2127.
Mahmood T, Mehnaz S, Fleischmann F, Ali R, Hashmi ZH, Iqbal Z. 2014. Soil sterilization effects on root growth and formation of rhizosheaths in wheat seedlings. *Pedobiologia* **57**(3): 123-130.
Malacrinò A, Karley AJ, Schena L, Bennett AE. 2020. Soil microbial diversity impacts plant microbiomes more than herbivory. *bioRxiv*: 2020.2009.2030.320317.
Maldonado-Gonzalez MM, Schiliro E, Prieto P, Mercado-Blanco J. 2015. Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. *Environmental Microbiology* **17**(9): 3139-3153.
Manrique AER, Mazzuchelli RdCL, Araujo ASF, Araujo FF. 2019. Conditioning and coating of *Urochloa brizantha* seeds associated with inoculation of *Bacillus subtilis*. *Pesquisa Agropecuária Tropical* **49**: e55536.
Mao TT, Chen XJ, Ding HX, Chen XY, Jiang XL. 2020. Pepper growth promotion and Fusarium wilt biocontrol by *Trichoderma hamatum* MHT1134. *Biocontrol Science and Technology* **30**(11): 1228-1243.
Marti G, Erb M, Boccard J, Glauser G, Doyen GR, Villard N, Robert CAM, Turlings TCJ, Rudaz S, Wolfender JL. 2013. Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. *Plant, Cell & Environment* **36**(3): 621-639.
Martin M. 2011. CUTADAPT removes adapter sequences from high-throughput sequencing reads. *EMBnet J* **17**(10–2): 10-12.
Martinez-Medina A, Flors V, Heil M, Mauch-Mani B, Pieterse CMJ, Pozo MJ, Ton J, van Dam NM, Conrath U. 2016. Recognizing plant defense priming. *Trends in Plant Science* **21**(10): 818-822.
Martinez-Viveros O, Jorquera MA, Crowley DE, Gajardo G, Mora ML. 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of Soil Science and Plant Nutrition* **10**(3): 293-319.
Massalha H, Korenblum E, Malitsky S, Shapiro OH, Aharoni A. 2017. Live imaging of root-bacteria interactions in a microfluidics setup. *Proceedings of the National Academy of Sciences of the United States of America* **114**(17): 4549-4554.
Mattiacci L, Dicke M, Posthumus MA. 1995. beta-Glucosidase - an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **92**(6): 2036-2040.
Mauch-Mani B, Baccelli I, Luna E, Flors V. 2017. Defense priming: an adaptive part of induced resistance. *Annual Review of Plant Biology* **68**: 485-512.
Mazzoleni S, Carteni F, Bonanomi G, Senatore M, Termolino P, Giannino F, Incerti G, Rietkerk M, Lanzotti V, Chiusano ML. 2015. Inhibitory effects of extracellular self-DNA: a general biological process? *New Phytologist* **206**(1): 127-132.
McMurdie PJ, Holmes S. 2013. phyloseq: an R rackage for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**(4): e61217.

Megali L, Glauser G, Rasmann S. 2014. Fertilization with beneficial microorganisms decreases tomato defenses against insect pests. *Agronomy for Sustainable Development* **34**(3): 649-656.
Megali L, Schlau B, Rasmann S. 2015. Soil microbial inoculation increases corn yield and insect attack. *Agronomy for Sustainable Development* **35**(4): 1511-1519.
Meiners SJ, Phipps KK, Pendergast TH, Canam T, Carson WP. 2017. Soil microbial communities alter leaf chemistry and influence allelopathic potential among coexisting plant species. *Oecologia* **183**(4): 1155-1165.
Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker P, et al. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* **332**(6033): 1097-1100.
Mhlongo MI, Piater LA, Steenkamp PA, Labuschagne N, Dubery IA. 2020. Metabolic profiling of PGPR-treated tomato plants reveal priming-related adaptations of secondary metabolites and aromatic amino acids. *Metabolites* **10**(5): 210.
Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**: 431-450.
Morcillo RJL, Singh SK, He DX, An G, Vilchez JI, Tang K, Yuan FT, Sun YZ, Shao CY, Zhang S, et al. 2020. Rhizobacterium-derived diacetyl modulates plant immunity in a phosphate-dependent manner. *EMBO Journal* **39**(2): e102602.
Moreira H, Pereira SIA, Marques A, Rangel A, Castro PML. 2019. Effects of soil sterilization and metal spiking in plant growth promoting rhizobacteria selection for phytotechnology purposes. *Geoderma* **334**: 72-81.
Morris WF, Hufbauer RA, Agrawal AA, Bever JD, Borowicz VA, Gilbert GS, Maron JL, Mitchell CE, Parker IM, Power AG, et al. 2007. Direct and interactive effects of enemies and mutualists on plant performance: a meta-analysis. *Ecology* **88**(4): 1021-1029.
Muci AL, Jorquera MA, Avila AI, Rengel Z, Crowley DE, Mora MD. 2012. A combination of cellular automata and agent-based models for simulating the root surface colonization by bacteria. *Ecological Modelling* **247**: 1-10.
Murphy JF, Zehnder GW, Schuster DJ, Sikora EJ, Polston JE, Kloepper JW. 2000. Plant growth-promoting rhizobacterial mediated protection in tomato against Tomato mottle virus. *Plant Disease* **84**(7): 779-784.
Naluyange V, Ochieno DMW, Maingi JM, Ornbori O, Mukaminega D, Amoding A, Odendo M, Okoth SA, Shivoga WA, Muoma JVO. 2014. Compatibility of *Rhizobium* inoculant and water hyacinth compost formulations in Rosecoco bean and consequences on *Aphis fabae* and *Colletotrichum lindemuthianum* infestations. *Applied Soil Ecology* **76**: 68-77.
Neal AL, Ahmad S, Gordon-Weeks R, Ton J. 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PLoS ONE* **7**(4): e35498.
Nelson EB. 2018. The seed microbiome: origins, interactions, and impacts. *Plant and Soil* **422**(1-2): 7-34.
Nilsson RH, Larsson K-H, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, et al. 2018. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research* **47**(D1): D259-D264.
O'Brien FJM, Dumont MG, Webb JS, Poppy GM. 2018. Rhizosphere bacterial communities differ according to fertilizer regimes and cabbage (*Brassica oleracea* var. capitata L.) harvest time, but not aphid herbivory. *Frontiers in Microbiology* **9**: 1620.
O'Callaghan M. 2016. Microbial inoculation of seed for improved crop performance: issues and opportunities. *Appl Microbiol Biotechnol* **100**(13): 5729-5746.
Ohgushi T. 2005. Indirect interaction webs: herbivore-induced effects through trait change in plants. *Annual Review of Ecology Evolution and Systematics* **36**: 81-105.
Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Suggests M. 2007. The vegan package. *Community ecology package* **10**(631-637): 719.
Ourry M, Lebreton L, Chaminade V, Guillerm-Erckelboudt AY, Herve M, Linglin J, Marnet N, Ourry A, Paty C, Poinsot D, et al. 2018. Influence of belowground

herbivory on the dynamics of root and rhizosphere microbial communities. *Frontiers in Ecology and Evolution* **6**: 91.

Palmer CM, Hindt MN, Schmidt H, Clemens S, Guerinot ML. 2013. *MYB10* and *MYB72* are required for growth under iron-limiting conditions. *PLoS Genetics* **9**(11): e1003953.

Pangesti N, Pineda A, Dicke M, van Loon JJA. 2015a. Variation in plant-mediated interactions between rhizobacteria and caterpillars: potential role of soil composition. *Plant Biology* **17**(2): 474-483.

Pangesti N, Pineda A, Pieterse CMJ, Dicke M, van Loon JJA. 2013. Two-way plant-mediated interactions between root-associated microbes and insects: from ecology to mechanisms. *Frontiers in Plant Science* **4**: 414.

Pangesti N, Reichelt M, van de Mortel JE, Kapsomenou E, Gershenzon J, van Loon JJA, Dicke M, Pineda A. 2016. Jasmonic acid and ethylene signaling pathways regulate glucosinolate levels in plants during rhizobacteria-induced systemic resistance against a leaf-chewing herbivore. *Journal of Chemical Ecology* **42**(12): 1212-1225.

Pangesti N, Vandenbrande S, Pineda A, Dicke M, Raaijmakers JM, van Loon JJA. 2017. Antagonism between two root-associated beneficial *Pseudomonas* strains does not affect plant growth promotion and induced resistance against a leaf-chewing herbivore. *FEMS Microbiology Ecology* **93**(4): fix038.

Pangesti N, Weldegergis BT, Langendorf B, van Loon JJA, Dicke M, Pineda A. 2015b. Rhizobacterial colonization of roots modulates plant volatile emission and enhances the attraction of a parasitoid wasp to host-infested plants. *Oecologia* **178**(4): 1169-1180.

Papadopoulou GV, van Dam NM. 2017. Mechanisms and ecological implications of plant-mediated interactions between belowground and aboveground insect herbivores. *Ecological Research* **32**(1): 13-26.

Pastor V, Luna E, Mauch-Mani B, Ton J, Flors V. 2013. Primed plants do not forget. *Environmental and Experimental Botany* **94**(Supplement C): 46-56.

Paulson JN, Stine OC, Bravo HC, Pop M. 2013. Differential abundance analysis for microbial marker-gene surveys. *Nature Methods* **10**(12): 1200-1205.

Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology* **11**(11): 789-799.

Pieterse CMJ, Dicke M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science* **12**(12): 564-569.

Pieterse CMJ, van der Does D, Zamioudis C, Leon-Reyes A, van Wees SCM. 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology* **28**: 489-521.

Pieterse CMJ, van Wees SCM, Hoffland E, van Pelt JA, van Loon LC. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8**(8): 1225-1237.

Pieterse CMJ, van Wees SCM, van Pelt JA, Knoester M, Laan R, Gerrits N, Weisbeek PJ, van Loon LC. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* **10**(9): 1571-1580.

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, van Wees SCM, Bakker P. 2014. Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology* **52**: 347-375.

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S, Crecchio C. 2015. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertility of Soils* **51**(4): 403-415.

Pineda A, Kaplan I, Bezemer TM. 2017. Steering soil microbiomes to suppress aboveground insect pests. *Trends in Plant Science* **22**(9): 770-778.

Pineda A, Kaplan I, Hannula SE, Ghanem W, Bezemer TM. 2020. Conditioning the soil microbiome through plant-soil feedbacks suppresses an aboveground insect pest. *New Phytologist* **226**(2): 595-608.

Pineda A, Soler R, Weldegergis BT, Shimwela MM, van Loon JJA, Dicke M. 2013. Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. *Plant, Cell & Environment* **36**(2): 393-404.

Pineda A, Zheng SJ, van Loon JJA, Dicke M. 2012. Rhizobacteria modify plant-aphid interactions: a case of induced systemic susceptibility. *Plant Biology* **14**: 83-90.

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

Poelman EH, Broekgaarden C, van Loon JJA, Dicke M. 2008. Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology* **17**(14): 3352-3365.

Poelman EH, Kessler A. 2016. Keystone herbivores and the evolution of plant defenses. *Trends in Plant Science* **21**(6): 477-485.

Poveda J, Jimenez-Gomez A, Saati-Santamaria Z, Usategui-Martin R, Rivas R, Garcia-Fraile P. 2019. Mealworm frass as a potential biofertilizer and abiotic stress tolerance-inductor in plants. *Applied Soil Ecology* **142**: 110-122.

Price PW, Bouton CE, Gross P, McPheron BA, Thompson JN, Weis AE. 1980. Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annual Review of Ecology and Systematics* **11**(1): 41-65.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**(D1): D590-D596.

R Core Team 2018. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2015.

Raaijmakers JM, Leeman M, Vanoorschot MMP, Vandersluis I, Schippers B, Bakker P. 1995. Dose-response relationships in biological-control of *Fusarium*-wilt of radish by *Pseudomonas* spp. *Phytopathology* **85**(10): 1075-1081.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moenne-Loccoz Y. 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* **321**(1-2): 341-361.

Raj N, Shetty NP, Shetty HS. 2004. Seed bio-priming with *Pseudomonas fluorescens* isolates enhances growth of pearl millet plants and induces resistance against downy mildew. *International Journal of Pest Management* **50**(1): 41-48.

Ran LX, van Loon LC, Bakker P. 2005. No role for bacterially produced salicylic acid in rhizobacterial induction of systemic resistance in *Arabidopsis*. *Phytopathology* **95**(11): 1349-1355.

Rasmann S, Bennett A, Biere A, Karley A, Guerrieri E. 2017. Root symbionts: powerful drivers of plant above- and belowground indirect defenses. *Insect Science* **24**(6): 947-960.

Rasmann S, Erwin AC, Halitschke R, Agrawal AA. 2011. Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *Journal of Ecology* **99**(1): 16-25.

Rasmann S, Kollner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* **434**(7034): 732-737.

Rasmann S, Turlings TCJ. 2016. Root signals that mediate mutualistic interactions in the rhizosphere. *Current Opinion in Plant Biology* **32**: 62-68.

Rilling JI, Acuna JJ, Nannipieri P, Cassan F, Maruyama F, Jorquera MA. 2019. Current opinion and perspectives on the methods for tracking and monitoring plant growth-promoting bacteria. *Soil Biology & Biochemistry* **130**: 205-219.

Rocha I, Ma Y, Souza-Alonso P, Vosatka M, Freitas H, Oliveira RS. 2019. Seed coating: a tool for delivering beneficial microbes to agricultural crops. *Frontiers in Plant Science* **10**: 1357

Roessingh P, Stadler E, Fenwick GR, Lewis JA, Nielsen JK, Hurter J, Ramp T. 1992. Oviposition and tarsal chemoreceptors of the cabbage root fly are stimulated by

glucosinolates and host plant-extracts. *Entomologia Experimentalis et Applicata* **65**(3): 267-282.
Rolfe SA, Griffiths J, Ton J. 2019. Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. *Current Opinion in Microbiology* **49**: 73-82.
Romera FJ, Garcia MJ, Lucena C, Martinez-Medina A, Aparicio MA, Ramos J, Alcantara E, Angulo M, Perez-Vicente R. 2019. Induced systemic resistance (ISR) and Fe deficiency responses in dicot plants. *Frontiers in Plant Science* **10**: 287.
Ruan JJ, Zhou YX, Zhou ML, Yan J, Khurshid M, Weng WF, Cheng JP, Zhang KX. 2019. Jasmonic acid signaling pathway in plants. *International Journal of Molecular Sciences* **20**(10): 2479.
Rubin RL, van Groenigen KJ, Hungate BA. 2017. Plant growth promoting rhizobacteria are more effective under drought: a meta-analysis. *Plant and Soil* **416**(1-2): 309-323.
Rusman Q, Lucas-Barbosa D, Poelman EH, Dicke M. 2019. Ecology of plastic flowers. *Trends in Plant Science* **24**(8): 725-740.
Rybakova D, Mancinelli R, Wikstrom M, Birch-Jensen AS, Postma J, Ehlers RU, Goertz S, Berg G. 2017. The structure of the *Brassica napus* seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome* **5**: 104.
Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiology* **134**(3): 1017-1026.
Ryu CM, Hu CH, Locy RD, Kloepper JW. 2005. Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. *Plant and Soil* **268**(1-2): 285-292.
Sanchez-Bayo F, Wyckhuys KAG. 2019. Worldwide decline of the entomofauna: a review of its drivers. *Biological Conservation* **232**: 8-27.
Santhanam R, Groten K, Meldau DG, Baldwin IT. 2014. Analysis of plant-bacteria interactions in their native habitat: bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages. *PLoS ONE* **9**(4): e94710.
Santos F, Penaflor M, Pare PW, Sanches PA, Kamiya AC, Tonelli M, Nardi C, Bento JMS. 2014. A novel interaction between plant-beneficial rhizobacteria and roots: colonization induces corn resistance against the root herbivore *Diabrotica speciosa*. *PLoS ONE* **9**(11): e113280.
Saravanakumar D, Lavanya N, Muthumeena B, Raguchander T, Suresh S, Samiyappan R. 2008. *Pseudomonas fluorescens* enhances resistance and natural enemy population in rice plants against leaffolder pest. *Journal of Applied Entomology* **132**(6): 469-479.
Sarkar D. 2008. *Lattice: multivariate data visualization with R*. New York: Springer & Business Media.
Sasse J, Martinoia E, Northen T. 2018. Feed your friends: do plant exudates shape the root microbiome? *Trends in Plant Science* **23**(1): 25-41.
Schoene WJ. 1916. The cabbage root maggot: its biology and control. *New York Agricultural Experiment Station* **419**.
Schoonhoven LM, van Loon JJA, Dicke M. 2005. *Insect-plant biology*. Oxford, U.K.: Oxford University Press.
Schultz JC, Appel HM, Ferrieri AP, Arnold TM. 2013. Flexible resource allocation during plant defense responses. *Frontiers in Plant Science* **4**: 324.
Schutz L, Gattinger A, Meier M, Muller A, Boller T, Mader P, Mathimaran N. 2018. Improving crop yield and nutrient use efficiency via biofertilization-a global meta-analysis. *Frontiers in Plant Science* **8**: 2204.
Segarra G, van der Ent S, Trillas I, Pieterse CMJ. 2009. MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biology* **11**(1): 90-96.
Sekar J, Raju K, Duraisamy P, Vaiyapuri PR. 2018. Potential of Finger millet indigenous rhizobacterium *Pseudomonas* sp MSSRFD41 in Blast disease management-growth

promotion and compatibility with the resident rhizomicrobiome. *Frontiers in Microbiology* **9**: 1029.

Sharifi R, Ryu CM 2017. Chatting with a tiny belowground member of the holobiome: communication between plants and growth-promoting rhizobacteria. In: Becard G ed. *How Plants Communicate with Their Biotic Environment*. London: Academic Press Ltd-Elsevier Science Ltd, 135-160.

Shavit R, Ofek-Lalzar M, Burdman S, Morin S. 2013. Inoculation of tomato plants with rhizobacteria enhances the performance of the phloem-feeding insect *Bemisia tabaci*. *Frontiers in Plant Science* **4**: 306.

Singh S, Singh UB, Malviya D, Paul S, Sahu PK, Trivedi M, Paul D, Saxena AK. 2020. Seed biopriming with microbial inoculant triggers local and systemic defense responses against *Rhizoctonia solani* causing banded leaf and sheath blight in maize (*Zea mays* l.). *International Journal of Environmental Research and Public Health* **17**(4): 1396.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* **67**(10): 4742-4751.

Soler R, Bezemer TM, Cortesero AM, Van der Putten WH, Vet LEM, Harvey JA. 2007. Impact of foliar herbivory on the development of a root-feeding insect and its parasitoid. *Oecologia* **152**(2): 257-264.

Sripontan Y, Tan CW, Hung MH, Young CC, Hwang SY. 2014. Effects of plant-growth-promoting microorganisms and fertilizers on growth of cabbage and tomato and *Spodoptera litura* performance. *Journal of Asia-Pacific Entomology* **17**(3): 587-593.

Stam JM, Kroes A, Li YH, Gols R, van Loon JJA, Poelman EH, Dicke M. 2014. Plant interactions with multiple insect herbivores: from community to genes. *Annual Review of Plant Biology* **65**: 689-713.

Strigul NS, Kravchenko LV. 2006. Mathematical modeling of PGPR inoculation into the rhizosphere. *Environmental Modelling & Software* **21**(8): 1158-1171.

Stringlis IA, de Jonge R, Pieterse CMJ. 2019. The age of coumarins in plant-microbe interactions. *Plant and Cell Physiology* **60**(7): 1405-1419.

Stringlis IA, Yu K, Feussner K, de Jonge R, van Bentum S, Van Verk MC, Berendsen RL, Bakker P, Feussner I, Pieterse CMJ. 2018. *MYB72*-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proceedings of the National Academy of Sciences of the United States of America* **115**(22): E5213-E5222.

Szczech M, Shoda M. 2006. The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*. *Journal of Phytopathology* **154**(6): 370-377.

Talekar NS, Shelton AM. 1993. Biology, ecology, and management of the diamondback moth. *Annual Review of Entomology* **38**: 275-301.

Taylor A, Allen P, Bennett M, Bradford K, Burris J, Misra M. 1998. Seed enhancements. *Seed Science Research* **8**(2): 245-256.

Thuler RT, Iost FH, Charlo HCD, de Bortoli SA. 2017. Assessment of bio-priming of seeds for germination and growth of onion (*Allium cepa* l.) and cabbage (*Brassica oleracea* var. capitata l.) seeds. *Revista Colombiana De Entomologia* **43**(2): 195-200.

Tkacz A, Bestion E, Bo ZY, Hortala M, Poole PS. 2020. Influence of plant fraction, soil, and plant species on microbiota: a multikingdom comparison. *mBio* **11**(1): e02785-02719.

Torres-Cortes G, Bonneau S, Bouchez O, Genthon C, Briand M, Jacques MA, Barret M. 2018. Functional microbial features driving community assembly during seed germination and emergence. *Frontiers in Plant Science* **9**: 902.

Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2015. Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environmental Microbiology Reports* **7**(1): 40-50.

Tsukanova KA, Chebotar VK, Meyer JJM, Bibikova TN. 2017. Effect of plant growth-promoting Rhizobacteria on plant hormone homeostasis. *South African Journal of Botany* **113**: 91-102.

Turan M, Ekinci M, Yildirim E, Gunes A, Karagoz K, Kotan R, Dursun A. 2014. Plant growth-promoting rhizobacteria improved growth, nutrient, and hormone content of cabbage (*Brassica oleracea*) seedlings. *Turkish Journal of Agriculture and Forestry* **38**(3): 327-333.

Turlings TCJ, Erb M. 2018. Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. *Annual Review of Entomology* **63**: 433-452.

Tytgat TOG, Verhoeven KJF, Jansen JJ, Raaijmakers CE, Bakx-Schotman T, McIntyre LM, van der Putten WH, Biere A, van Dam NM. 2013. Plants know where it hurts: root and shoot jasmonic acid induction elicit differential responses in *Brassica oleracea*. *PLoS ONE* **8**(6): e65502.

Utsumi S, Ando Y, Roininen H, Takahashi J, Ohgushi T. 2013. Herbivore community promotes trait evolution in a leaf beetle via induced plant response. *Ecology Letters* **16**(3): 362-370.

Vadassery J, Reichelt M, Hause B, Gershenzon J, Boland W, Mithofer A. 2012. CML42-mediated calcium signaling coordinates responses to *Spodoptera* herbivory and abiotic stresses in *Arabidopsis*. *Plant Physiology* **159**(3): 1159-1175.

Valenzuela-Soto JH, Estrada-Hernandez MG, Ibarra-Laclette E, Delano-Frier JP. 2010. Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards whitefly *Bemisia tabaci* development. *Planta* **231**(2): 397-410.

van Bel M, Diels T, Vancaester E, Kreft L, Botzki A, Van de Peer Y, Coppens F, Vandepoele K. 2018. PLAZA 4.0: an integrative resource for functional, evolutionary and comparative plant genomics. *Nucleic Acids Research* **46**(D1).

van Dam NM, Bouwmeester HJ. 2016. Metabolomics in the rhizosphere: tapping into belowground chemical communication. *Trends in Plant Science* **21**(3): 256-265.

van de Mortel JE, de Vos RCH, Dekkers E, Pineda A, Guillod L, Bouwmeester K, van Loon JJA, Dicke M, Raaijmakers JM. 2012. Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiology* **160**(4): 2173-2188.

van den Bosch TJM, Welte CU. 2020. The microbial diversity of cabbage pest *Delia radicum* across multiple life stages. *Frontiers in Microbiology* **11**: 315.

van der Ent S, Verhagen BWM, van Doorn R, Bakker D, Verlaan MG, Pel MJC, Joosten RG, Proveniers MCG, van Loon LC, Ton J, et al. 2008. *MYB72* is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiology* **146**(3): 1293-1304.

van der Heijden MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* **11**(3): 296-310.

van der Putten WH, Bardgett RD, Bever JD, Bezemer TM, Casper BB, Fukami T, Kardol P, Klironomos JN, Kulmatiski A, Schweitzer JA, et al. 2013. Plant-soil feedbacks: the past, the present and future challenges. *Journal of Ecology* **101**(2): 265-276.

van der Putten WH, Vet LEM, Harvey JA, Wackers FL. 2001. Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. *Trends in Ecology & Evolution* **16**(10): 547-554.

van Geem M, Harvey JA, Cortesero AM, Raaijmakers CE, Gols R. 2015. Interactions between a belowground herbivore and primary and secondary root metabolites in wild cabbage. *Journal of Chemical Ecology* **41**(8): 696-707.

van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J. 2006. Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **103**(14): 5602-5607.

van Loon LC, Bakker P, van der Heijdt WHW, Wendehenne D, Pugin A. 2008. Early responses of tobacco suspension cells to rhizobacterial elicitors of induced systemic resistance. *Molecular Plant-Microbe Interactions* **21**(12): 1609-1621.

van Oosten VR, Bodenhausen N, Reymond P, van Pelt JA, van Loon LC, Dicke M, Pieterse CMJ. 2008. Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. *Molecular Plant-Microbe Interactions* **21**(7): 919-930.

van Veen JA, van Overbeek LS, van Elsas JD. 1997. Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* **61**(2): 121-135.

van Zandt PA, Agrawal AA. 2004. Community-wide impacts of herbivore-induced plant responses in milkweed (*Asclepias syriaca*). *Ecology* **85**(9): 2616-2629.

Vandesompele J, De Preter K, Pattyn F, Poppe B, van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**(7): 0034.0031.

Velten S, Leventon J, Jager N, Newig J. 2015. What is sustainable agriculture? A systematic review. *Sustainability* **7**(6): 7833-7865.

Verhagen BWM, Glazebrook J, Zhu T, Chang HS, van Loon LC, Pieterse CMJ. 2004. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Molecular Plant-Microbe Interactions* **17**(8): 895-908.

Verma V, Ravindran P, Kumar PP. 2016. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology* **16**: 86.

Wang JJ, Li QQ, Xu S, Zhao W, Lei Y, Song CH, Huang ZY. 2018. Traits-based integration of multi-species inoculants facilitates shifts of indigenous soil bacterial community. *Frontiers in Microbiology* **9**: 1692.

Wang MG, Ruan WB, Kostenko O, Carvalho S, Hannula SE, Mulder PPJ, Bu FJ, van der Putten WH, Bezemer TM. 2019. Removal of soil biota alters soil feedback effects on plant growth and defense chemistry. *New Phytologist* **221**(3): 1478-1491.

Wang SH, Voorrips RE, Steenhuis-Broers G, Vosman B, van Loon JJA. 2016. Antibiosis resistance against larval cabbage root fly, *Delia radicum*, in wild *Brassica*-species. *Euphytica* **211**(2): 139-155.

Weston DJ, Pelletier DA, Morrell-Falvey JL, Tschaplinski TJ, Jawdy SS, Lu TY, Allen SM, Melton SJ, Martin MZ, Schadt CW, et al. 2012. *Pseudomonas fluorescens* induces strain-dependent and strain-independent host plant responses in defense networks, primary metabolism, photosynthesis, and fitness. *Molecular Plant-Microbe Interactions* **25**(6): 765-778.

Wickham H, Averick M, Bryan J, Chang W, D'Agostino McGowan L, François R, Grolemund G, Hayes A, Henry L, Hester J. 2019. Welcome to the Tidyverse. *Journal of Open Source Software* **4**(43): 1686.

Wintermans PCA, Bakker P, Pieterse CMJ. 2016. Natural genetic variation in *Arabidopsis* for responsiveness to plant growth-promoting rhizobacteria. *Plant Molecular Biology* **90**(6): 623-634.

Witzel K, Strehmel N, Baldermann S, Neugart S, Becker Y, Becker M, Berger B, Scheel D, Grosch R, Schreiner M, et al. 2017. *Arabidopsis thaliana* root and root exudate metabolism is altered by the growth-promoting bacterium *Kosakonia radicincitans* DSM 16656(T). *Plant and Soil* **419**(1-2): 557-573.

Wright DA, Zydenbos SM, Wessman P, O'Callaghan M, Townsend RJ, Jackson TA, van Koten C, Mansfield S. 2017. Surface coating aids survival of *Serratia entomophila* (Enterobacteriaceae) in granules for surface application. *Biocontrol Science and Technology* **27**(12): 1383-1399.

Wu K, Fang ZY, Guo R, Pan B, Shi W, Yuan SF, Guan HL, Gong M, Shen B, Shen QR. 2015. Pectin enhances bio-control efficacy by inducing colonization and secretion of secondary metabolites by *Bacillus amyloliquefaciens* SQY 162 in the rhizosphere of tobacco. *PLoS ONE* **10**(5): e0127418.

Yang J, Kloepper JW, Ryu CM. 2009. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science* **14**(1): 1-4.

Yang JW, Yi HS, Kim H, Lee B, Lee S, Ghim SY, Ryu CM. 2011. Whitefly infestation of pepper plants elicits defence responses against bacterial pathogens in leaves and roots and changes the below-ground microflora. *Journal of Ecology* **99**(1): 46-56.

Yi YL, de Jong A, Frenzel E, Kuipers OP. 2017. Comparative transcriptomics of *Bacillus mycoides* strains in response to potato-root exudates reveals different genetic adaptation of endophytic and soil isolates. *Frontiers in Microbiology* **8**: 1487.

Yuan J, Chaparro JM, Manter DK, Zhang RF, Vivanco JM, Shen QR. 2015a. Roots from distinct plant developmental stages are capable of rapidly selecting their own microbiome without the influence of environmental and soil edaphic factors. *Soil Biology & Biochemistry* **89**: 206-209.

Yuan J, Zhang N, Huang QW, Raza W, Li R, Vivanco JM, Shen QR. 2015b. Organic acids from root exudates of banana help root colonization of PGPR strain *Bacillus amyloliquefaciens* NJN-6. *Scientific Reports* **5**: 13438.

Zalucki MP, Shabbir A, Silva R, Adamson D, Liu SS, Furlong MJ. 2012. Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): just how long is a piece of string? *Journal of Economic Entomology* **105**(4): 1115-1129.

Zamioudis C, Hanson J, Pieterse CMJ. 2014. beta-Glucosidase BGLU42 is a *MYB72*-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in *Arabidopsis* roots. *New Phytologist* **204**(2): 368-379.

Zamioudis C, Mastranesti P, Dhonukshe P, Blilou I, Pieterse CMJ. 2013. Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. bacteria. *Plant Physiology* **162**(1): 304-318.

Zehnder G, Kloepper J, Yao CB, Wei G. 1997. Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria. *Journal of Economic Entomology* **90**(2): 391-396.

Zeileis A, Hothorn T 2002. Diagnostic checking in regression relationships. R News **2**(3): 7-10.

Zekveld C, Markham J. 2011. Exposure to aphids increases alder growth and nitrogen fixation. *Botany* **89**(4): 255-261.

Zhang HM, Xie XT, Kim MS, Kornyeyev DA, Holaday S, Pare PW. 2008. Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. *Plant Journal* **56**(2): 264-273.

Zhang N, Wang DD, Liu YP, Li SQ, Shen QR, Zhang RF. 2014. Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. *Plant and Soil* **374**(1-2): 689-700.

Zhou WJ, Qin SJ, Lyu DG, Zhang P. 2015. Soil sterilisation and plant growth-promoting rhizobacteria promote root respiration and growth of sweet cherry rootstocks. *Archives of Agronomy and Soil Science* **61**(3): 361-370.

Zhu F, Heinen R, van der Sluijs M, Raaijmakers C, Biere A, Bezemer TM. 2018. Species-specific plant-soil feedbacks alter herbivore-induced gene expression and defense chemistry in *Plantago lanceolata*. *Oecologia* **188**(3): 801-811.

Zytynska SE, Eicher M, Rothballer M, Weisser WW. 2020. Microbial-mediated plant growth promotion and pest suppression varies under climate change. *Frontiers in Plant Science* **11**: 573578.

Summary

Soils contain a multitude of microbes, including plant pathogens as well as microbes that benefit plant health. The beneficial microbes include root-colonizing bacteria that can promote plant growth. These bacteria also can trigger a resistance mechanism called induced systemic resistance, which provides the whole plant with a faster and stronger response when the plant is attacked. This thesis explores the effects of adding a specific rhizobacterium on plant growth and plant resistance against herbivorous insects. By using cabbage, *Brassica oleracea*, this thesis aims to investigate (1) plant responses to addition of the rhizobacterium *Pseudomonas simiae* WCS417r, and (2) how common cabbage-associated insect herbivores respond to the bacterial addition *via* plant-mediated effects.

Chapter 2 presents a literature review on the bi-directional effects of plant-insect-rhizobacteria interactions. Although previous literature reviews have discussed rhizobacterial effects on plant growth, few explicitly focused on plant resistance against insects. This review explored how herbivorous insects and their communities are affected by rhizobacterial addition through modification of plant resistance. In addition, the literature study includes a novel focus to the field on how insect feeding affects root-associated bacterial communities.

Although rhizobacteria have shown to increase plant growth and/or resistance against an herbivorous insect, only a few crops and insect species have so far been investigated. In chapter 3, I expand the knowledge on plant responses and plant resistance against herbivorous insects when the soil has been inoculated with rhizobacteria. After growing cabbage (*B. oleracea*) in sterilized or *P. simiae*-inoculated soil, I infested the plants with larvae of the cabbage moth *Mamestra brassicae*, the diamondback moth *Plutella xylostella*, or the cabbage root fly *Delia radicum*. The rhizobacterial soil inoculation resulted in an increased aboveground plant biomass compared to biomass of plants growing in non-inoculated soil. Furthermore, the inoculation affected insect performance. Inoculation decreased *P. xylostella* biomass and increased *D. radicum* biomass, when insects fed on plants grown on rhizobacterially inoculated soil, compared to plants grown on non-inoculated soil. *Mamestra brassicae* biomass was similar on plants grown on inoculated and non-inoculated soil. Taken together, these results indicate that insect herbivores have species-specific responses when feeding on plants growing in rhizobacteria-inoculated soil.

Instead of mixing rhizobacteria into the soil, or pouring a rhizobacterial solution on the soil, the bacteria can be disseminated together with the seed. This can be done through biopriming: the seeds are hydrated together with a bacterium in liquid, but removed from the

liquid before the seed radicle appears. This method has rarely been considered with respect to plant-insect interactions. I assessed *B. oleracea*'s growth and resistance against herbivorous insects, when grown from seeds bacterized with *P. simiae* (Chapter 4). Aboveground plant biomass was similar between plants grown from bacterized seeds and water-soaked seeds. The performance of larvae of *M. brassicae* and *P. xylostella* was similar when feeding on plants grown from either bacterized seeds or water-soaked seeds. No conclusions could be drawn on the performance of *D. radicum*, when the insects were feeding on plants grown from bacterized seeds or water-soaked seeds. These results indicate that the choice of method for rhizobacterial dissemination is influencing the effect on cabbage plant growth. Furthermore, compared to soil inoculation, rhizobacterial bacterization of seeds may affect plant-insect interactions to a lesser extent.

In chapter 5, I explored the rhizobacterial plant resistance modification of *B. oleracea* using co-feeding by two insect herbivores and soil inoculation in a factorial experiment. Here, the shoot feeder *P. xylostella* and the root feeder *D. radicum* were simultaneously attacking the same host plant. The plants were grown in sterilized soil with inoculated *P. simiae*. The inoculation increased plant aboveground biomass compared to biomass of plants grown in non-inoculated soil. Furthermore, inoculation decreased the measured consumed leaf area by *P. xylostella* compared to consumed leaf area from plants grown on non-inoculated soil. However, insect biomass was similar when feeding on inoculated or non-inoculated plants. *Plutella xylostella* biomass decreased when co-feeding with *D. radicum*, regardless of rhizobacterial soil inoculation. The extent of root damage by *D. radicum* feeding singly was similar as after co-feeding with *P. xylostella*. From these results I conclude that rhizobacterial soil inoculation can influence the consumed leaf area by the herbivore.

The final experimental chapter, chapter 6, focused on rhizosphere microbial alterations after herbivore infestation and rhizobacterium inoculation, and how these community alterations affect the growth and resistance of plants growing consecutively in the conditioned soil. *Brassica oleracea* plants were infested with the herbivores *Brevicoryne brassicae*, *P. xylostella*, *D. radicum*, or *D. radicum* plus *P. simiae* soil inoculation, or only with *P. simiae* soil inoculation. The rhizosphere was sampled after two weeks of plant growth and soil conditioning. After the plants and insects were removed, the soil was used to grow a new set of *B. oleracea* plants. The second set of plants were assessed for growth and resistance against *D. radicum*. A principle component analysis clustered the microbiome mainly into groups according to herbivore feeding location,

i.e. the shoot- and root feeders, but did not separate the inoculated plants' microbiome from the control plants' microbiome. Assessment of the second set of plants showed that soil conditioning affected plant growth and resistance in a treatment-specific way, but not according to previous herbivore shoot- or root feeding location. The results suggest that specific members, and not the complete microbial community, are responsible for alterations in the plant defense.

This thesis contributes to fundamental as well as applied knowledge of how rhizobacterium addition to plants affects plant growth and defense against herbivorous insects, with a focus on cabbage, *B. oleracea*. Taken together, the research presented shows a context dependency of the effects of rhizobacterium addition on plant growth promotion, as well as rhizobacterial increase in plant resistance to insects: the method of rhizobacterial dissemination and the identity of the herbivorous insect affect the rhizobacterial effects on insects *via* plant-mediated effects. In general, measuring the outcomes of insect-plant-microbe interactions is complex and time-consuming, calling for simplifications such as utilizing specialized plant growth media. Yet for a successful rhizobacterium application to promote crop health within agriculture, more knowledge is required from field studies. Many answers to questions on how and when to add microbes are still unknown. However, such answers are needed to optimize bacterial plant growth promotion for many species and environments, particularly answers regarding soil type and soil nutrient status will be instrumental to optimize bacteria-assisted agriculture.

Samenvatting

Bodems bevatten een veelvoud aan microben, waaronder plantpathogenen en microben die de gezondheid van planten ten goede komen. Deze nuttige microben zijn onder meer wortel-bewonende bacteriën ofwel rhizobacteriën die de groei van planten kunnen bevorderen. Naast het verbeteren van de plantengroei, kunnen verschillende van deze bacteriesoorten de systemische plantafweer tegen aanvallers verbeteren. Deze bacteriën activeren een mechanisme, geïnduceerde systemische resistentie, waardoor de hele plant een snellere en sterkere afweerreactie vertoont wanneer de plant wordt aangevallen. Dit proefschrift onderzoekt de effecten van het toevoegen van een specifieke rhizobacterie op plantengroei en plantresistentie tegen plantenetende insecten. Door het gebruik van kool, *Brassica oleracea*, is het doel van dit proefschrift de reacties van planten op verschillende methoden van toevoeging van de rhizobacterie *Pseudomonas simiae* stam WCS417r te onderzoeken, zowel als de reacties van algemeen op koolplanten voorkomende insecten op de bacteriële toevoeging via plant-gemedieerde effecten.

Hoofdstuk 2 presenteert een literatuuronderzoek naar de bi-directionele effecten van plant-insect-rhizobacteriële interacties. Hoewel eerdere literatuuroverzichten de effecten van rhizobacteriën op plantengroei hebben besproken, waren er maar weinig die expliciet gericht waren op de afweer van planten tegen insecten. In dit overzicht werd onderzocht hoe herbivore insecten en insectengemeenschappen worden beïnvloed door toevoeging van rhizobacteriën en de effecten daarvan op de afweer van planten. Daarnaast bevat de literatuurstudie ook een nieuwe focus op hoe voedselopname van insecten de rhizobacteriële gemeenschap beïnvloedt.

Hoewel is aangetoond dat rhizobacteriën de plantengroei en/of resistentie tegen een herbivoor insect verhogen, zijn tot nu toe slechts enkele gewassen en insectensoorten onderzocht. In hoofdstuk 3 vergroot ik de kennis over plantresponsen en plantafweer tegen herbivore insecten met betrekking tot rhizobacteriële bodeminoculatie. Na het kweken van koolplanten in gesteriliseerde of met *P. simiae* geïnoculeerde bodem, stelde ik de planten bloot aan vraat door larven van de kooluil *Mamestra brassicae*, de koolmot *Plutella xylostella* of de koolwortelvlieg *Delia radicum*. De toevoeging van rhizobacteriën aan de bodem resulteerde in een verhoogde bovengrondse plantenbiomassa in vergelijking met biomassa van niet-geïnoculeerde planten. Bovendien had de rhizobacteriële inoculatie invloed op de groei van insecten. Inoculatie verminderde de biomassa van *P. xylostella* en verhoogde de biomassa van *D. radicum*, wanneer insecten aten van rhizobacterieel geïnoculeerde

planten in vergelijking met niet-geïnoculeerde planten. De biomassa van *Mamestra brassicae* was vergelijkbaar op geïnoculeerde en niet-geïnoculeerde planten. Alles bij elkaar geven deze resultaten aan dat herbivore insecten soort-specifieke reacties laten zien wanneer ze zich voeden met planten geïnoculeerd met rhizobacteriën in de bodem.

In plaats van rhizobacteriën door de grond te mengen of een rhizobacteriële oplossing op de grond te gieten, kunnen de bacteriën samen met het zaad worden verspreid. Dit kan door middel van 'biopriming': de zaden worden samen met een bacterie in vloeistof gehydrateerd, maar uit de vloeistof gehaald voordat de kiemwortel verschijnt. Deze methode is zelden overwogen met betrekking tot interacties tussen planten en insecten. Ik heb de groei en afweer van *B. oleracea* tegen herbivore insecten onderzocht, wanneer deze was opgekweekt uit zaden geïnoculeerd met *P. simiae* (Hoofdstuk 4). Bovengrondse plantenbiomassa was vergelijkbaar tussen planten die waren gekweekt uit met bacteriën in water behandelde zaden en met alleen water doordrenkte zaden. De groei van de larven van *M. brassicae* en *P. xylostella* was vergelijkbaar wanneer ze aten van planten die waren gekweekt uit met bacteriën behandelde zaden of met water doordrenkte zaden. Er konden geen conclusies worden getrokken over de groei van *D. radicum*, nadat deze zich voedde met planten die waren opgekweekt uit met bacteriën behandelde zaden of met water doordrenkte zaden. Deze resultaten geven aan dat de keuze van de methode voor de verspreiding van rhizobacteriën het effect op de groei van koolplanten beïnvloedt. Bovendien lijkt rhizobacteriële inoculatie van zaden, in vergelijking met bodeminoculatie, de interacties tussen planten en insecten in mindere mate te beïnvloeden.

In hoofdstuk 5 heb ik de rhizobacteriële modificatie van plantafweer van *B. oleracea* onderzocht wanneer twee insecten zich op de plant voedden na bodeminoculatie, in een factoriële proefopzet. Hier vielen de bladvreter *P. xylostella* en de wortelvreter *D. radicum* tegelijkertijd dezelfde waardplant aan. De planten werden gekweekt in gesteriliseerde grond waaraan *P. simiae* was toegevoegd. De inoculatie verhoogde de bovengrondse biomassa van planten in vergelijking met de biomassa van planten die in niet-geïnoculeerde grond werden gekweekt. Bovendien verminderde inoculatie het geconsumeerde bladoppervlak door *P. xylostella* in vergelijking met het geconsumeerde bladoppervlak van planten die op niet-geïnoculeerde grond waren gekweekt. Het insectengewicht was echter vergelijkbaar na het voeden op geïnoculeerde of niet-geïnoculeerde planten. Het gewicht van *P. xylostella* nam af bij gelijktijdige voeding met *D. radicum*, ongeacht de rhizobacteriële bodeminoculatie. De wortelschade veroorzaakt door *D. radicum* was

vergelijkbaar voor gelijktijdige bladvraat door *P. xylostella* en alleen wortelvraat door *D. radicum*. Uit deze resultaten concludeer ik dat rhizobacteriële bodeminoculatie de hoeveelheid bladvraat kan beïnvloeden.

Het laatste experimentele hoofdstuk, hoofdstuk 6, ging over microbiële veranderingen in de rhizosfeer na blootstelling aan herbivoren en inoculatie met rhizobacteriën, en hoe deze veranderingen in de microbiële gemeenschap vervolgens de groei van koolplanten beïnvloeden. *Brassica oleracea*-planten werden blootgesteld aan de herbivoren *Brevicoryne brassicae*, *P. xylostella*, *D. radicum* of *D. radicum* samen met een inoculatie van *P. simiae*, of alleen geïnoculeerd met *P. simiae*. De rhizosfeer werd bemonsterd na twee weken plantengroei of bodeminoculatie. Nadat de planten en insecten waren verwijderd, werd de grond gebruikt om een nieuwe set *B. oleracea*-planten te laten groeien. De tweede set planten werd beoordeeld op groei en resistentie tegen *D. radicum*. Een principiële componentenanalyse clusterde het microbioom voornamelijk in groepen gerelateerd aan de voedingslocatie, dwz spruit- of wortelvraat, maar leverde geen onderscheid op tussen het microbioom van de geïnoculeerde planten en dat van de controleplanten. Beoordeling van de tweede set planten toonde aan dat bodemconditionering de plantengroei en -afweer op een behandelingsspecifieke manier beïnvloedde, en niet op basis van de voedingslocatie. De resultaten suggereren dat specifieke leden, en niet de volledige microbiële gemeenschap, verantwoordelijk zijn voor veranderingen in de plantafweer.

Dit proefschrift draagt bij aan zowel fundamentele als toegepaste kennis over hoe de toevoeging van een rhizobacterie de plantengroei en de afweer tegen herbivore insecten beïnvloedt, met een focus op kool, *B. oleracea*. Alles bij elkaar genomen toont het onderzoek dat in dit proefschrift wordt gepresenteerd, een contextafhankelijkheid aan van de toevoeging van een rhizobacterie aan de bevordering van plantengroei en toename van plantresistentie tegen insecten: de methode van de inoculatie van rhizobacteriën en de identiteit van het herbivoor insect beïnvloeden de rhizobacteriële effecten op insecten via plantgemedieerde effecten. In het algemeen is het meten van de interactie tussen insecten, planten en microben complex en tijdrovend, waardoor vereenvoudigingen nodig zijn, zoals het gebruik van gespecialiseerde groeimedia voor planten. Maar voor een succesvolle toepassing van rhizobacteriën om de gezondheid van landbouwgewassen te bevorderen, is meer kennis uit veldonderzoek nodig. Veel vragen over hoe en wanneer microben moeten worden toegevoegd, zijn nog onbeantwoord en meer kennis is nodig om de groei van planten voor veel soorten en omgevingen te optimaliseren.

Informatie over de invloed van abiotische omgevingsfactoren, zoals bodemsoorten, en biotische factoren, zoals plant-geassocieerde insecten of microbiële gemeenschappen, zal in de toekomst essentieel zijn om de gewasopbrengst te verhogen.

Sammanfatting

Jord innehåller en mängd mikrober, inklusive växtpatogener och goda mikrober som gynnar växtens allmänna hälsa. Dessa gynsamma mikrober inkluderar rotkoloniserande bakterier som kan främja växtens utveckling. Förutom att öka tillväxten kan flera av dessa arter av bakterier förbättra växtens försvar mot angripare. Bakterierna utlöser en mekanism, så kallad inducerad systemisk resistens, som ger hela plantan ett snabbare och starkare försvar när växten attackeras. Denna avhandling undersöker hur tillsatsen av en specifik bakterie påverkar växtens utveckling och dess resistens mot angripande insekter. Genom att använda kål (*Brassica oleracea*) syftar denna avhandling till att undersöka (1) växters respons på tillsatts av rotbakterien *Pseudomonas simiae* WCS417r, och (2) hur vanliga med kål förknippade växtätande insekter reagerar på bakterietinokuleringen *via* växtförmedlade förändringar.

Kapitel 2 presenterar en litteraturöversikt över dubbelriktade effekter i interaktionen mellan växter-insekter-rotbakterier. Även om tidigare litteraturgranskningar har diskuterat bakteriella effekter på växtens utveckling, fokuserar få på växtförsvar mot insekter. Denna litteraturstudie undersökte hur växtätande insekter och insektssamhällen påverkas av bakteriell berikning genom växten. Dessutom tillför litteraturstudien ett nytt fokus till kunskapsfältet om på hur insektsangrepp påverkar rotförknippade bakteriesamhällen.

Även om rotbakterier har visat sig öka växtens utveckling och/eller motståndskraft mot växtätande insekter, har endast ett fåtal växt- och insektsarter hittills undersökts i detta sammanhang. I kapitel 3 breddar jag kunskapen om växtresponser och -resistans mot angripande insekter i samband med rotbakteriell jordinokulering. Efter att ha odlat kål (*B. oleracea*) i steriliserad eller *P. simiae*-inokulerad jord infekterade jag växterna med larver av kålfly (*Mamestra brassicae*), kålmal (*Plutella xylostella*) eller kålfluga (*Delia radicum*). Den bakteriella inokuleringen resulterade i en ökad växtbiomassa ovan jord jämfört med växtbiomassa från växter som växte i icke-inokulerad jord. Dessutom påverkade den bakteriella inokuleringen insekternas utveckling. Inokuleringen minskade *P. xylostella*s biomassa och ökade *D. radicum*s biomassa när insekter konsumerade växter som växt i rhizobakteriellt inokulerade jord, jämfört med växter som växt i icke-inokulerad jord. *Mamestra brassicae*s biomassa skiljde sig inte mellan inokulerade och icke-inokulerade växter. Sammantaget tyder dessa resultat på att växtätande insekter har en artspecifik respons när de konsumerar bakteriellt inokulerade växter.

Istället för att blanda bakterier i jorden, eller väta jorden med en bakterie-lösning, kan bakterierna spridas tillsammans med fröet. Detta

kan göras genom biopriming där fröna hydratiseras tillsammans med en bakterier i en vätska, men fröet avlägsnas från vätskan innan frösträngen dyker upp. Denna metod har sällan undersökts med avseende på växter-insektsinteraktioner. Jag studerade *B. oleracea*s tillväxt och försvar mot växtätande insekter, när de odlades från frön med biopriming *P. simiae* (kapitel 4). Växtbiomassa ovan jord var likartad mellan växter som odlats från bakterie-behandlade frön och vattendränkta frön. Utvecklingen för *M*. *brassicae* och *P. xylostella* larver var likartad när de konsumerade växter som odlats från antingen bakterie-behandlade eller vattendränkta frön. Inga slutsatser kunde dras från utvecklingen för *D. radicum* när insekterna konsumerade växter som odlats av bakterie-behandlade eller vattendränkta frön. Dessa resultat indikerar att valet av den bakteriella spridningsmetoden påverkar kålväxtens tillväxt. Vidare, jämfört med jordinokulering, verkar rotbakteriell bakterie-behandlade av frön påverka växter-insektsinteraktioner i mindre utsträckning.

I kapitel 5 undersökte jag modifiering av växtresistensförmåga från rotbakterier med hjälp av två växtätande insekter som samtidigt konsumerar samma *B. oleracea* planta, tillsammans med jordinokulering i ett faktoriellt experiment. Här attackerade larver av skott-ätaren *P. xylostella* och rot-ätaren *D. radicum* samma värdväxt. Växterna odlades i steriliserad jord inokulerad med *P. simiae*. Inokuleringen ökade växtbiomassa ovan jord jämfört med växtbiomassa ovan jord från växter som odlats på icke-inokulerad jord. Inokuleringen minskade *P. xylostella*s konsumerad bladyta jämfört med *P. xylostella*s konsumerad bladyta från växter som odlats på icke-inokulerad jord. Insektsvikten var dock samma vid konsumering av växter från inokulerad eller icke-inokulerad jord. *P. xylostella*s vikt minskade när insekterna levde av samma planta som *D. radicum*, oavsett bakteriell jordinokulering. Skadorna från *D. radicum* var jämförbara vare sig de konsumerade samtidigt med *P. xylostella* på samma värdväxt, eller när de levde själva på växten. Från dessa resultat drar jag slutsatsen att rotbakteriell jordinokulering kan påverka den konsumerade bladmängden.

Det sista experimentella kapitlet, kapitel 6, fokuserade på mikrobiella förändringar i rhizosfären efter växtätares angrepp och rhizobacterium-inokulering, och hur dessa förändringar påverkar följande växts tillväxt. *Brassica oleracea* växter infekterades med kålbladlusen *Brevicoryne brassicae*, *P. xylostella*, *D. radicum* eller *D. radicum* tillsammans med en inokulering av *P. simiae*, eller endast med *P. simiae*. Rhizosfären togs ut och analyserades efter två veckors tillväxt och konditionering av jorden. Efter att växterna och insekterna hade avlägsnats användes jorden för att odla en ny uppsättning av kål. Den andra uppsättningen växter bedömdes med avseende på utveckling och

resistens mot *D. radicum*. En huvudkomponentanalys grupperade mikrobiomen huvudsakligen i relation till insektens lokalisering, skott- och rot-konsumenter, men separerade inte de inokulerade växterna från kontrollplantornas mikrobiom. Bedömning av den andra uppsättningen växter visade att konditionering av jorden påverkade växternas utveckling och resistens på ett behandlingsspecifikt sätt och inte beroende på insektens lokalisering. Resultaten tyder på att specifika medlemmar i rhizosfären, till skillnad från hela mikrobiella beståndet, leder till förändringar i växtförsvaret.

Denna avhandling, med fokus på kål *B. oleracea*, bidrar till grundläggande såväl som tillämpad kunskap om hur tillsatsen av rotbakterier påverkar tillväxt och resistens mot växtätande insekter. Sammantaget visar forskningen som presenteras i avhandlingen ett beroendeförhållandet mellan bakterieberikning och växtens utveckling, likaväl som bakterieinokulering och ökning av växtresistens mot insekter: metoden för rhizobakteriell spridning och identiteten på den växtätande insekten påverkar de bakteriella effekterna på insekter *via* växtförmedlade effekter. Generellt är mätning av insekt-växt-mikrob-interaktion komplex och tidskrävande, vilket kräver förenklingar som att använda specialiserade rotningsmedier. För en framgångsrik berikningsapplicering för att främja grödors utveckling inom agrikultur, krävs mer kunskap från fältstudier. Många svar till frågor om hur och när berikningen ska ske med mikrober är fortfarande obesvarade. Dessa svar behövs för att optimera användningen av bakterier, framförallt svar angående jord typer och jordens näringsinnehåll, kommer att vara avgörande i framtiden för bakterie-assisterat jordbruk.

Acknowledgements

To do a PhD has always been a sort of dream to me. Over the years, I made some tentative tries on different careers but found myself going back to the academic sector time after time. After my Masters' graduation, slowly the dream came alive. After working for several years I could finally embark on a PhD project. Throughout the PhD project I had help from many people which I here want to specifically thank.

First, I would like to thank my supervisors. Thank you Marcel for all your feedback on the many versions of different texts I sent you, and for guiding the review towards being accepted for publication. Joop, your kind humor always cheered me up. Thank you for your feedback on my texts, as well as all other advice that you have given me. I'm thankful for you letting me try a couple of wild experimental ideas. Ana, thank you for your feedback and advice.

This thesis could not have been completed without the support from the staff members of the Ento rearing team, I thank you all. Pieter, your consideration of the needs of other people is amazing.

During the thesis several students worked alongside me. Mike, Rocky, Thomas, Malav, you are all great people, and I'm lucky that you wanted to work with me. Thank you for what you taught me, and your patience with me as I developed into a better teacher and individual. Julian, I greatly enjoyed working with you, and I hope that we will keep in touch for a very long time ahead. I admire your understanding of insects and plants. I can't wait to hear what your next adventure will be!

I'm grateful for the support from my colleagues. Thank you to the whole Laboratory of Entomology, I hope I don't forget anyone. Angelique, Peter de Jong, Sander, Jeroen, Erik, Hans, Rieta, Els, Luuk, Bram, Hanneke, Yidong, Yavanna, Julian, Katherine, Mariska, Mitchel, Thibault, Maximillien, Tessa, Anna, Helena, Max, Maite, Simone, Fillippo, Alexander, Karen, Yvonne, Martine, Gabriela, Davy, Daan, Kay, Marilyn, Kelly, Gerard, Julien, Sandeep, Stijn, Parth, Eveline, Kyra, Alessia, Qi, Aidan, Helen, Pedro, Shaphan, Marcela… All of you contribute to making an irresistible and great atmosphere. Also, thank you all the "older" members of the family such as Eddie, Lucille, Quint, Tim, Chantal, Jessica, Feng, Jeltje, Ilka, Antonio, Emma, Jitte, Shuhang, I had fun times with you all.

Thank you Jeroen "the flientist", I look forward to see where life takes you. Thank you Maite, the way you tackle any problem is impressive. I much enjoyed working with you. Max, having you as my colleague is absolutely great. You are one of the funniest guys I know, you made me laugh until I cried on so many occasions. Thank you for your humor and kindness. Daan, thank you for always making time for my questions! I owe you so many favors, I will never be able to pay them

all back. You are a most wonderful teacher, and always seem to have some amazing insights to share. Kay, I had great fun going to the Vienna conference with you. Thank you for your advices, I use them regularly.

Liana and Gabe, thanks for helping with my texts! I don't think I will ever get tired of talking with you guys. I hope to enjoy many dinners and games with you in the future.

Tessa, you lighted up my day, and you always make me and others feel appreciated. You made a deep impression on me, I hope to adopt some of your sunny life attitude. Also, I hope to enjoy more of your cooking!

Yidong, thank you for all the happy hours we spent together, for all the long discussions and laughter. If the science career doesn't work out for you, I hope that you go for a career in design and scientific art instead.

Janneke, you make everyone feel appreciated. I can ask and discuss anything with you without feeling stupid, from work-related questions to the big problems in life. Thank you for always taking time to help and listen to others. Not only have I loved to work with you, I had the luxury of spending time with you outside work.

Peter, I don't even know where to start, I have so many thing I'd like to thank you for. Without you, this book and the whole project would have been very hard indeed. During the years you have gone from my colleague to my best friend. You make me a better person by showing me all the different ways.

Outside of work, I'd like to thank Camiel van Loon. You have helped by advising me how to reduce my leg and back pain, and getting rid of a lot of work related stress.

Thanks to my buddies Tor and Henrik, for being interested and asking all the questions about my work.

I want to thank my family for all your support! My father, Claes, thank you for all the phone-calls in which you patiently listened to my explanations and my experiments. My mother Sylvia, thanks for bringing me to all your classes when I was small, I'm sure that's why I like it so much in academia. With your help, I may have become somewhat of a decent writer. Mika, thank you for all discussions and word explanations, you have made my argumentation better. Siri, thank you for your support! You show me how to increase my patience for the writing process, and help me understand otherwise (for me) strange human behavior.

Thank you to all other friends and family, you inspire me.

//Jag vill tacka min familj för all er support! Farsan, tack för alla telefonsamtal där du lyssnar snällt på mina förklaringar till alla mina experiment. Mamma, tack för att du tog med mig till alla klassrummen när jag var liten, jag är säker på att det är därför jag tris så bra i akedemin. Med din hjälp har jag blivit en hyffsad skrivare. Mika, tack för alla diskussioner och ordförklaringar, med din hjälp har jag kunnat vässa mina argument. Siri, tack för ditt stöd! Du viar mig hur jag ska ha mer tålamod med mitt skrivade och hur jag ska tolka mänsliga beteenden som jag inte alltid förstår.

Tack alla andra vänner till vår familj som har inspirerat mig.

Daniel, utan dig skulle inte den här boken finnas.
Daniel, without you this book would not exist.

Publication list

Fernández de Bobadilla MF, Friman J, Pangesti N, Dicke M, van Loon JJA, Pineda A. 2017. Does drought stress modify the effects of plant-growth promoting rhizobacteria on an aboveground chewing herbivore? *Insect Science* **24**(6): 1034-1044.

Friman J, Pineda A, Gershenzon J, Dicke M, van Loon JJA. 2021a. Differential effects of the rhizobacterium *Pseudomonas simiae* on above- and belowground chewing insect herbivores. *Journal of Applied Entomology* **145**(3): 250-260.

Friman J, Pineda A, van Loon JJA, Dicke M. 2021b. Bidirectional plant-mediated interactions between rhizobacteria and shoot-feeding herbivorous insects: a community ecology perspective. *Ecological Entomology* **46**(1): 1–10.

About the author

Julia Friman was born on 31 December 1982 in Gothenburg, Sweden, and grew up both in the city and in the countryside, where she fell in love with plants. She received her primary education in 2000 from the public gymnasium of Mölnlycke outside of Gothenburg. After a detour into literature studies at Växsjö University, Växsjö, Sweden, (books and texts being another of Julia's passions), in 2006 she started studying biology at Gothenburg University. For a summer month, she took part in a wild-life survey of predators in the northern Swedish Alps. She conducted her education's minor thesis at Syngenta Seeds, Landskrona, Sweden, where she worked on fine-mapping resistance genes against root aphids in sugar beets. The major thesis was conducted at BIM Kemi, Gothenburg, where she investigated black mold on plasterboards. She also enjoyed an internship at the County administrative board of Västra Götaland, where she mainly contributed to a survey of giant trees in the county. She obtained a master's degree in Plant ecology in 2012. After her graduation, she moved to Amsterdam, the Netherlands. In 2016, she started her Ph.D. training at the Laboratory of Entomology, Wageningen, under the supervision of Prof. dr. Marcel Dicke and Prof. dr. Joop J.A. van Loon.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Julia Friman
Date: 28 June 2021
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
▶ **First presentation of your project**		
Presentation PhD meeting	08-04-2016	1.5
▶ **Writing or rewriting a project proposal**		
Use of beneficial rhizobacteria against herbivore pests in Brassica crops: modulating factors and underlying mechanisms	10-01-2016	6.0
▶ **Writing a review or book chapter**		
Friman, J. et al. Bidirectional plant-mediated interactions between rhizobacteria and shoot-feeding herbivorous insects: a community ecology perspective, Ecological Entomology (2021), 46(1): 1-10, DOI: 10.1111/een.12966	2016-2021	6.0
Subtotal Start-Up Phase		13.5

2) Scientific Exposure	date	cp
▶ **EPS PhD student days**		
EPS PhD Get2Gether	15-02-2018 - 16-02-2018	0.6
EPS PhD Get2Gether	11-02-2019 - 12-02-2019	0.6
▶ **EPS theme symposia**		
EPS Theme 2 Symposium & Willie Commelin Scholten Day - Interactions between Plants and Biotic Agents	22-01-2016	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten Day - Interactions between Plants and Biotic Agents	24-02-2018	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten Day - Interactions between Plants and Biotic Agents	01-02-2019	0.3
▶ **Lunteren Days and other national platforms**		
NEV Entomologendag	15-12-2017	0.3
NEV Entomologendag	13-12-2019	0.3
Annual Meeting Experimental Plant Sciences	08-04-2019 - 09-04-2019	0.6
▶ **Seminars (series), workshops and symposia**		
Yearly Entomology Laboratory Research Exchange Meeting	10-06-2015	0.3
Yearly Entomology Laboratory Research Exchange Meeting	01-06-2016	0.3
Yearly Entomology Laboratory Research Exchange Meeting	24-05-2018	0.3
Yearly Entomology Laboratory Research Exchange Meeting	07-06-2019	0.3
WEES seminar: Yolanda Chen	21-05-2015	0.1
WEES seminar: Arnold van Vliet, Take the few steps to mass media exposure.	21-01-2016	0.1
Plant Soil Interaction seminars: Oene Oenema, What chemical and physical soil properties should every researcher measure?	20-04-2016	0.1
Plant Soil Interaction seminars: Paula Harkes, The effect of alien plant species on the rhizosphere food web & Janna Barel, Legacy effects of cover crop mixtures in rotation.	20-09-2016	0.1
Plant Soil Interaction seminars: Johannes Postma, How do architectural traits influence resource acquisition and plant growth: Stimulation studies with OpenSimRoot.	17-02-2017	0.1
Plant Soil Interaction seminars: Annelein Meisner, Coping with extremes: Drought.	03-05-2017	0.1

	Plant Soil Interaction seminars: Wopke van der Werf, How intercropping can enhance crop ecosystem services in agricultural systems.	25-09-2017	0.1
	Plant microbe interactions workshop, NIOO, Wageningen, the Netherlands	30-06-2016	0.3
	14th Workshop on Plant-Insect Interactions, Amsterdam, the Netherlands	14-11-2019	0.3
►	**Seminar plus**		
	WEES workshop: Yolanda Chen.	21-05-2015	0.1
	WEES workshop: Arnold van Vliet, Take the few steps to mass media exposure.	21-01-2016	0.1
►	**International symposia and congresses**		
	67th International Symposium on Crop Protection, Ghent, Belgium	19-5-2015	0.3
	Future IPM in Europe 3.0, Riva del Garda, Italy	17-09-2017 - 20-08-2017	0.9
	LabLinks: Good Germs – Bad Germs, Ghent, Belgium	05-10-2018	0.3
	European Plant Science Retreat (EPSR), Utrecht, the Netherlands	03-07-2018 - 06-07-2018	1.0
	Microbe-assisted Crop Production (MiCROPe), Vienna, Austria	02-12-2019 - 05-12-2019	1.2
►	**Presentations**		
	Future IPM in Europe 3.0 (poster), Riva del Garda, Italy	17-20-2017	1.0
	NEV Entomologendag (poster), Wageningen, the Netherlands	15-12-2017	1.0
	EPS theme 2 symposium, Amsterdam, the Netherlands	24-01-2018	1.0
	EPSR (poster), Utrecht, the Netherlands	03-07-2018	0.0
	MiCROPe, Vienna, Austria	04-12-2019	1.0
►	**IAB interview**		
►	**Excursions**		
	Subtotal Scientific Exposure		13.7

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
Basic Statistics (PE&RC & SENSE), Wageningen, the Netherlands	11-12-2017 - 19-12-2017	1.5
DIMPA: Detection and Identification of Microorganisms in Plants and Arthropods, Umeå, Sweden	08-10-2018 - 11-10-2018	0.9
► **Individual research training**		
Study trip, INRA University of Rennes, France	01-07-2019 - 02-07-2019	0.6
Subtotal In-Depth Studies		3.0

4) Personal Development	*date*	*cp*
► **General skill training courses**		
Project and Time Management (Wageningen Graduate Schools)	06-02-2018 - 20-03-2018	1.5
Writing Grant Proposals (Wageningen in'to Languages)	01-10-2019 - 26-11-2019	2.0
Scientific Writing (Wageningen in'to Languages)	15-04-2019 - 11-06-2019	1.8
The Choice: Un-box your PhD process & take charge of your performance (Wageningen Graduate Schools)	13-01-2020 - 17-02-2020	0.5
► **Organisation of meetings, PhD courses or outreach activities**		
Wageningen PhD Symposium	26-04-2016	1.5
Subtotal Personal Development		7.3

TOTAL NUMBER OF CREDIT POINTS*	**37.5**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	
** A credit represents a normative study load of 28 hours of study.*	

The research presented in this thesis was performed at the Laboratory of Entomology of Wageningen University & Research (WUR) and was supported by private funding.

Cover design and thesis layout by Julia Friman
Printed by GVO drukkers en vormgevers B.V., Ede, The Netherlands