Rhizobacterial modification of plant defenses against insect herbivores:

From molecular mechanisms to tritrophic interactions

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To my parents Waryanti & Nursabani

TABLE OF CONTENTS

1

General introduction

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INTRODUCTION

Plants function as primary producers in terrestrial ecosystems and are exposed to a wide variety of organisms including detrimental and beneficial organisms. Moreover, plants also function as an element connecting below- and aboveground organisms, such as microbes inhabiting the roots and aboveground insects feeding on the shoots (Van der Putten *et al.*, 2001; Wardle *et al.*, 2004; Pineda *et al.*, 2010). An important group of organisms are belowground microbes that inhabit the rhizosphere. Interactions between plants and their root-associated microbiomes are important for plant fitness, and the crucial role of the root-associated microbes in plant function is reflected by the flow of up to 40% of the plant-produced carbon to the ectorhizosphere and rhizoplane, depending on plant species, age and environmental conditions (Lynch and Whipps, 1990). It has been proposed that the rhizospheric microbes serve as an extension of plant genomes, that plants can access whenever needed (Berendsen *et al.*, 2012; Rout and Southworth, 2013; Turner *et al.*, 2013). For instance, increasing plant access to phosphate and nitrogen is known to shape mutualistic interactions between plants and mycorrhizae and rhizobia (Breuillin *et al.*, 2010; Hoeksema *et al.*, 2010; Bonneau *et al.*, 2013). Moreover, a wide variety of beneficial microbes that reside in the rhizosphere are able to increase plant growth and immunity via a mechanism called induced systemic resistance (ISR), which is effective against various types of plant pathogenic microbes (Pieterse *et al.*, 1998; Van Loon *et al.*, 1998; Van Wees *et al.*, 1999; Ahn *et al.*, 2007; Pozo *et al.*, 2008). Beneficial microbes can also trigger physiological changes in the plant that have systemic effects on aboveground insects from different trophic levels (Gehring and Bennett, 2009; Leitner *et al.*, 2010; Pineda *et al.*, 2010; Katayama *et al.*, 2011; Pineda *et al.*, 2013). While the ecology of plant-mediated interactions between belowground beneficial microbes and aboveground insects has been widely studied, the knowledge on underlying molecular mechanisms involved in these multitrophic interactions is still limited and therefore needs to be further explored. Integration of ecological and molecular approaches is crucial for a better understanding of factors regulating these multitrophic interactions, and can yield practical applications for improving plant immunity and productivity.

Main objective and research questions:

The main objective of my PhD research was to further our understanding on mechanisms involved in the interactions between rhizobacteria, plants and leaf-chewing

insects. I have used a combination of gene transcriptional, chemical, insect performance and behavioral approaches and have addressed the following overall research questions:

1. How does rhizobacterial colonization affect plant direct and indirect defense to leaf-chewing insects?

2. What is the role of plant hormones, in particular the jasmonic acid (JA) and ethylene (ET)- signaling pathways and crosstalk between these pathways in modulating gene transcriptional responses and the synthesis of defensive compounds in rhizobacteria-mediated ISR against leaf-chewing insects?

STUDY SYSTEM

Arabidopsis thaliana Col-0 is a model plant species belonging to the Brassicaceae and has been widely used to study molecular and physiological mechanisms of plant interactions with microbes and insect herbivores employing different feeding modes (Pieterse *et al.*, 1998; Reymond *et al.*, 2004; De Vos *et al.*, 2005; Thatcher *et al.*, 2009; Verhage *et al.*, 2011; Zamioudis *et al.*, 2013). In the study of plant-insect interactions, the use of *A. thaliana* Col-0 has contributed to unraveling signaling pathways and the biosynthesis of secondary metabolites underlying plant direct and indirect defense to both specialist and generalist leaf-chewing and phloem-feeding insects (Van Poecke *et al.*, 2001; Van Poecke and Dicke, 2002; Reymond *et al.*, 2004; Mewis *et al.*, 2005; Bodenhausen and Reymond, 2007; De Vos *et al.*, 2007; Beekwilder *et al.*, 2008; Verhage *et al.*, 2011; Vos *et al.*, 2013; Zhang *et al.*, 2013). This model plant has also been used to explore molecular mechanisms involved in the effects of beneficial microbes on plant defense against pathogenic microbes (Pieterse *et al.*, 1998; Van Wees *et al.*, 1999; Ryu *et al.*, 2004; Ahn *et al.*, 2007; Van der Ent *et al.*, 2008) and to insect herbivores (Van Oosten *et al.*, 2008; Pineda *et al.*, 2012; Van de Mortel *et al.*, 2012).

The model system investigated is presented in Figure 1. The insect herbivores *Mamestra brassicae* and *Pieris brassicae* are folivores on brassicaceous plants. The generalist *M. brassicae* L. (Lepidoptera: Noctuidae; Cabbage moth) is distributed throughout most of Europe and Asia (Cartea *et al.*, 2014). The larvae are highly polyphagous, and feed on more than 70 plant species from 22 families. Eggs are laid in clutches of up to approximately 200 eggs, but the hatched larvae do not feed gregariously (Goulson and Cory, 1995; Chougule *et al.*, 2008). The specialist *P. brassicae* L. (Lepidoptera:

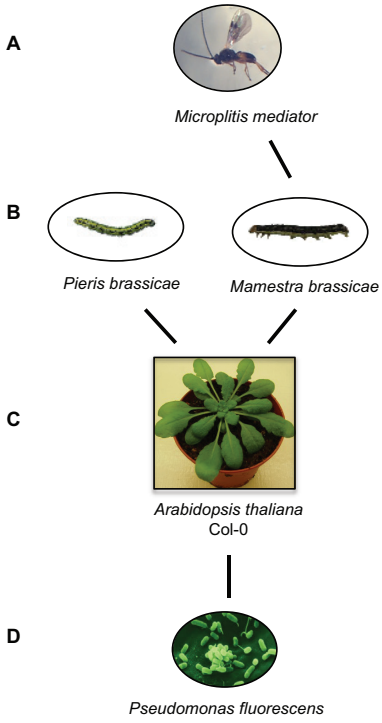

Figure 1. Model system used in this thesis consists of organisms from different trophic levels. A, insect parasitoid *Microplitis mediator*; B, caterpillar of the specialist *Pieris brassicae*; caterpillar of the generalist *Mamestra brassicae*; C, *Arabidopsis thaliana* Col-0; D, root-associated bacteria *Pseudomonas fluorescens*.

Pieridae; Large Cabbage White butterfly) is a common agricultural pest in Europe, North Africa and Asia causing significant damage to plants in the Brassicaceae (Feltwell, 1982). This specialist lays large batches of up to 150 eggs, and the first to third instar larvae feed gregariously. The fourth and fifth instars disperse to complete their development individually (Lemasurier, 1994). The insect parasitoid *Microplitis mediator* is one of the most important natural enemies of *M. brassicae* caterpillars (Lauro *et al.*, 2005), and it is known to parasitize ca. 40 species of lepidopteran herbivores (Li *et al.*, 2006a; Li *et al.*, 2006b). This parasitoid is a polyphagous solitary larval endoparasitoid that parasitizes first to third larval instars of *M. brassicae* (Malcicka and Harvey, 2014).

Pseudomonas fluorescens strains WCS417r and SS101 (abbreviated as *Pf.* WCS417r and *Pf.* SS101) are the rhizobacteria used to study molecular mechanisms of plant interactions with beneficial microbes with consequences for the defenses against pathogenic microbes and insect herbivores (Pieterse *et al.*, 1998; Van Loon *et al.*, 1998; Van Wees *et al.*, 1999; Ahn *et al.*, 2007; Pozo *et al.*, 2008; Van de Mortel *et al.*, 2012). The *Pf.* WCS417r and SS101 were isolated from the rhizosphere of wheat grown in soil suppressive against take-all disease (Lamers, 1988; De Souza *et al.*, 2003). *Pf.* WCS417r is able to colonize diverse plant species such as wheat, *A. thaliana* Col-0, carnation, radish and tomato (Van Loon *et al.*, 1998), whereas *Pf.* SS101 is known to colonize wheat, *A. thaliana* Col-0, and tomato (De Souza *et al.*, 2003; Mazzola *et al.*, 2007; Tran *et al.*, 2007; Van de Mortel *et al.*, 2012).

THESIS OUTLINE

Chapter 2: This chapter presents the state of the art. Belowground microorganisms and aboveground insects can interact bi-directionally via plant-mediated mechanisms. This chapter reviews the literature on how single micobe species and a community of root-associated microbes influences plant interactions with aboveground insects at different trophic levels. Furthermore, the effects of foliar herbivory by insects from different feeding guilds on interactions between plants and root-associated microbes are discussed. Finally, the role of phytohormones in coordinating plant growth and defense is reviewed in the context of microbe-plant-insect interactions.

Chapter 3: This chapter addresses the plant-mediated effects of the rhizobacterium *Pf.* WCS417r on the performance of the generalist *M. brassicae* and the specialist *P. brassicae,* as well as the underlying mechanisms. To unravel the underlying mechanisms, the expression of several plant defense-associated genes was assessed and their role further confirmed using a mutant impaired in JA-biosynthesis. The effect of soil composition on the strength of ISR caused by rhizobacterial colonization on the generalist caterpillar was evaluated.

Chapter 4: Here, rhizobacterium-plant-insect interactions were investigated by assessing the role of the JA-regulated MYC2 branch and the JA/ET-regulated ORA59 branch of the JA signal transduction pathway in regulating ISR against the generalist *M. brassicae*. Gene transcription, chemistry and performance of *M. brassicae* have been investigated for wild type *A. thaliana* Col-0 plants and mutants defective in the JA pathway, i.e. *dde2-2* and *myc2*, in the ET pathway, i.e. *ein2-1*, and in the JA/ET pathway, i.e. *ora59*.

Chapter 5: Most studies on microbe-plant-insect interactions mainly focus on interactions of plants with single species of beneficial microbes. In this chapter, two *P. fluorescens* strains, WCS417r and SS101, were used, which both are known to be able to trigger ISR. This was done to address the question how interactions between the two strains in the rhizosphere would affect the colonization of each other and the strength of ISR against the generalist caterpillar *M. brassicae*.

Chapter 6: While the effect of root-associated microbes on direct plant defense against insect herbivores has been studied previously, the effect of these microbes on indirect plant defense to herbivores is much less known. In this chapter, I explore how colonization by the rhizobacterium *Pf.* WCS417r affects indirect plant defense against

the generalist herbivore *M. brassicae*. This was done by investigating the behavior of the parasitoid *Microplitis mediator* and by analysis of the induced blend of plant volatiles and transcriptional responses of two terpene synthase genes, i.e. *TPS03* and *TPS04,* by comparing treatments of control plants versus rhizobacterial-colonized plants, upon feeding by the herbivore *M. brassicae*.

Chapter 7: Here, I discuss the most important findings of this thesis with a focus on topics relevant in microbe-plant-insect interactions, and suggest directions for future research.

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2

Two-way plant-mediated interactions between root-associated microbes and insects: from ecology to mechanisms

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ABSTRACT

Plants are members of complex communities and function as a link between above- and below-ground organisms. Associations between plants and soil-borne microbes commonly occur and have often been found beneficial for plant fitness. Root-associated microbes may trigger physiological changes in the host plant that influence interactions between plants and aboveground insects at several trophic levels. Aboveground, plants are under continuous attack by insect herbivores and mount multiple responses that also have systemic effects on belowground microbes. Until recently, both ecological and mechanistic studies have mostly focused on exploring these below- and above-ground interactions using simplified systems involving both single microbe and herbivore species, which is far from the naturally occurring interactions. Increasing the complexity of the systems studied is required to increase our understanding of microbe-plant-insect interactions and to gain more benefit from the use of non-pathogenic microbes in agriculture. In this review, we explore how colonization by either single non-pathogenic microbe species or a community of such microbes belowground affects plant growth and defense and how this affects the interactions of plants with aboveground insects at different trophic levels. Moreover, we review how plant responses to foliar herbivory by insects belonging to different feeding guilds affect interactions of plants with non-pathogenic soil-borne microbes. The role of phytohormones in coordinating plant growth, plant defenses against foliar herbivores while simultaneously establishing associations with non-pathogenic soil microbes is discussed.

Keywords: Insect herbivores, induced systemic resistance, mycorrhizae, plant growth promotion, phytohormones, parasitoids, rhizobacteria, rhizobia.

INTRODUCTION

Plants are members of complex communities and function as a link between above- and below-ground communities that consist of microbes, insects and other vertebrate and invertebrate animals (Bezemer and Van Dam, 2005; Dicke and Baldwin, 2010). In addition to a multitude of direct interactions between these different community members, indirect interactions occur via shared host plants (Ohgushi, 2005; Kaplan and Denno, 2007; Gehring and Bennett, 2009; Pineda *et al.*, 2010). To survive, plants need to optimally allocate resources to growth and defense (Herms and Mattson, 1992). For instance, in the presence of plant pathogens or insect herbivores, plants will allocate resources to the synthesis of defense compounds and as a consequence plant growth will decrease. Remarkably, plants form associations with non-pathogenic root-associated microbes such as mycorrhizae, rhizobia and rhizobacteria that can promote plant growth by increasing their access to soil minerals (Mendes *et al.*, 2011; Berendsen *et al.*, 2012; Bulgarelli *et al.*, 2013). Moreover, several species of non-pathogenic root-inhabiting microbes can trigger physiological changes and induction of defenses in the host plant that have systemic effects on aboveground insect communities involving organisms at several trophic levels (Leitner *et al.*, 2010; Pineda *et al.*, 2010; Katayama *et al.*, 2011b; Pineda *et al.*, 2013). Most studies in this area, however, mainly address plant interactions with single species of non-pathogenic microbes. In recent years, the root microbiome as a whole has appeared crucial for many aspects of plant development and immunity (Hol *et al.*, 2010; Mendes *et al.*, 2011; Partida-Martinez and Heil, 2011; Berendsen *et al.*, 2012; Martinuz *et al.*, 2012). Therefore, a shift should be made from studying single microbial species to investigating the community of root inhabiting microbes and its effects on plant–insect interactions.

Aboveground, plants are under continuous attack by various organisms such as insects and pathogens and mount multiple responses that have systemic effects on belowground microbes. Insect leaf chewing, for instance, leads to reduced leaf area and, therefore, reduced photosynthetic potential which may affect allocation of resources to the roots and the level of root exudation (Gehring and Bennett, 2009). Furthermore, induced plant defenses against plant pathogens or insect herbivores can alter concentrations of secondary metabolites in the shoots and roots that influence plant interactions with non-pathogenic soil microbes. During the past few years, evidence has accumulated that plants have a sophisticated defense mechanism by actively recruiting non-pathogenic root-associated microbes following attack by pathogens or insects (Rudrappa *et al.*, 2008; Lakshmanan *et al.*, 2012; Lee *et al.*, 2012b). By regulating its root secretion in

the form of carbon-rich exudates, plants can actually shape the root microbiome by affecting microbial diversity, density and activity (Barea *et al.*, 2005; Dennis *et al.*, 2010). More recently, significant progress has been made in understanding signaling pathways and molecules involved in recruitment of specific groups of microbes following foliar herbivory and defense activation (De Roman *et al.*, 2011; Yang *et al.*, 2011b; Yi *et al.*, 2011; Doornbos *et al.*, 2012; Lakshmanan *et al.*, 2012; Landgraf *et al.*, 2012; Lee *et al.*, 2012b; Neal *et al.*, 2012).

As sessile organisms, plants rely on a range of chemical compounds to repel enemies and attract mutualistic organisms above- and below-ground (Rasmann *et al.*, 2005; Dicke and Baldwin, 2010). The phytohormones jasmonic acid (JA) and salicylic acid (SA) function as major players in coordinating the complex signaling pathways involved in these multitrophic interactions (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). Other plant hormones such as ethylene (ET), abscisic acid (ABA), cytokinin (CK), gibberellin (GA) and auxin function as modulators of the hormone signaling backbone (Robert-Seilaniantz *et al.*, 2011; Meldau *et al.*, 2012; Pieterse *et al.*, 2012; Giron *et al.*, 2013). The underlying molecular pathways mediating plant-insect and plant-microbe interactions are interconnected. Induction of the JA- and SA- signaling pathways depends on the mode of feeding of the herbivorous insect species (De Vos *et al.*, 2005; Wu, 2010; Erb *et al.*, 2012; Thaler *et al.*, 2012; Soler *et al.*, 2013). In interactions between non-pathogenic rhizosphere microbes and plants, the phytohormones JA, SA and ET regulate symbiosis and mediate induced systemic resistance (ISR) elicited by several groups of non-pathogenic microbes (De Vleesschauwer *et al.*, 2009; Zamioudis and Pieterse, 2012). Moreover, recent experimental evidence has started to unveil the signaling pathways induced by root-associated microbes to stimulate plant growth. Here, we will review the role of these signaling pathways and their crosstalk in shaping microbe-plant-insect interactions. We have previously proposed that different groups of non-pathogenic microbes have similar plant-mediated effects on insect herbivores aboveground (Pineda *et al.*, 2010). Since then, the field of non-pathogenic microbe-plant-insect interactions has made significant advances. Here, we review those recent findings and outline future perspectives.

FROM EFFECTS OF MICROBES ON SINGLE HERBIVORE SPECIES TO EFFECTS ON INSECT COMMUNITIES

The field of microbe-plant-insect interactions has mainly addressed how a certain microbe affects single herbivore species. In nature, however, plants are sequentially or

simultaneously attacked by multiple herbivores, that in turn are attacked by parasitoids and predators. It is therefore not surprising that effects of non-pathogenic microbes on a specific herbivore species will depend on how such an herbivore is interacting with the community of herbivorous insects. For instance, colonization of four grass species by the mycorrhizal fungus *Rhizophagus irregularis* (formerly known as *Glomus intraradices*) leads to a significant increase in performance of the generalist caterpillar *Spodoptera littoralis* as well as in aboveground plant biomass (Kempel *et al.*, 2010). Interestingly, if the plants had been previously attacked by the same herbivore species, mycorrhization reduces the performance of a subsequent attacker as well as shoot biomass. The authors suggested that in herbivore-induced plants, mycorrhizal colonization mediates a shift of resource allocation from promoting plant growth to inducing resistance against insects. Whether plant signaling pathways are involved in this shift of resource allocation remains to be elucidated. In response to attack by multiple insect herbivores, plants activate different hormone signaling pathways depending on feeding characteristics of the insects (De Vos *et al.*, 2005; Li *et al.*, 2006). Recent studies show that induction of JA-dependent defenses against leaf chewers can be attenuated by previous infestation of phloem feeders such as aphids and whiteflies that activate the SA signaling pathway resulting in JA-SA antagonistic crosstalk mechanisms (Rodriguez-Saona *et al.*, 2010; Soler *et al.*, 2012; Zhang *et al.*, 2013). How non-pathogenic microbes can modify the interaction between multiple herbivores is a question that has not been explored so far.

From a multitrophic perspective, during the past few years several studies have addressed the effects of below-ground non-pathogenic microbes on third-trophic-level organisms i.e. arthropod predators and parasitoids, via changes in the emission of herbivore-induced plant volatiles (HIPVs) (Leitner *et al.*, 2010; Hoffmann *et al.*, 2011a,b,c; Katayama *et al.*, 2011a; Schausberger *et al.*, 2012; Ballhorn *et al.*, 2013; Pineda *et al.*, 2013). A set of studies with *Phaseolus vulgaris* bean plants showed that the mycorrhizal fungus *Glomus mosseae* resulted in reduction of spider-mite damage. In these studies mycorrhizae provided plants with a fitness benefit (i.e. increase of seed production) despite the increased performance of the herbivorous spider mite *Tetranychus urticae*, by enhancing the attraction and performance of predatory mites that feed on the spider mite (Hoffmann *et al.*, 2011a,b). Increased emission of β-ocimene and β-caryophyllene in mycorrhizal-colonized bean plants was associated with the attractiveness to the predatory mite (Schausberger *et al.*, 2012). However, root-associated microbes can also have negative plant-mediated effects on indirect plant defense. Colonization of *A. thaliana* roots by *Pseudomonas fluorescens* modified HIPV emission after infestation

by the generalist aphid *Myzus persicae* via JA-signaling and these changes reduced the attraction of the aphid parasitoid *Diaeretiella rapae* to the plants (Pineda *et al.*, 2013). Thus, non-pathogenic root-associated microbes can have positive or negative effects on the attraction of organisms at the third trophic level. Which molecular mechanisms are underlying these contrasting effects remains to be elucidated and may explain why in some interactions positive and in others negative effects on indirect plant defense occur.

In addition to the effects on plant volatiles, several root-colonizing microbes can also produce volatiles themselves. These microbial volatiles have a role in plant growth promotion and ISR against pathogens (Choudhary *et al.*, 2008; De Vleesschauwer *et al.*, 2009; Lee *et al.*, 2012a; Bulgarelli *et al.*, 2013; Zamioudis *et al.*, 2013). For instance, the short-chain volatile organic compound (VOC) 2,3-butanediol is produced by root-associated *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, and it can trigger ISR in *A. thaliana* against the pathogen *Erwinia carotovora* via the ET signaling pathway (Ryu *et al.*, 2004). Interestingly, 2,3-butanediol is also known as insect attractant (Bengtsson *et al.*, 2009; Del Pilar Marquez-Villavicencio *et al.*, 2011). Therefore, in addition to the indirect effects of microbes on herbivores via plant-mediated mechanisms, compounds produced by non-pathogenic root microbes could also have a direct effect on insect attraction. In this Research Topic, (Kupferschmied *et al.*, 2013) show insecticidal activity of some rhizobacteria-derived compounds. These direct effects of root-colonizing microbes on insect herbivores and their natural enemies need to be further assessed to gain a thorough understanding of their role in shaping plant-associated communities.

MOVING FROM EFFECTS OF SINGLE MICROBE SPECIES TO THE COMMUNITY OF ROOT-ASSOCIATED MICROBES

The microbe-plant interaction can start as early as the seed formation, e.g. many endophytes are transmitted to the seeds via the parental plant (Gundel *et al.*, 2011). Once the seed germinates in the soil, colonization of plant roots by multiple microbial species starts (Partida-Martinez and Heil, 2011). The majority of plant-associated microbes resides in the thin soil layer that is influenced by plant roots called rhizosphere, a dynamic niche in the soil that is strongly affected by the release of root exudates (Barea *et al.*, 2005; Lundberg *et al.*, 2012). The microbial community associated with plant roots, the so-called rhizosphere microbiome, has an important role in plant health and survival (Bakker *et al.*, 2013; Mendes *et al.*, 2013). The effects of the rhizosphere microbiome on

induced systemic resistance have mainly been studied for plant pathogen interactions (Mendes *et al.*, 2011), although mechanistic studies on the effects of the microbiome on ISR against herbivores have been initiated (Badri *et al.*, 2013). A study by (Hol *et al.*, 2010) demonstrated the importance of evaluating the soil microbiome as a whole when studying microbe plant insect interactions. This study showed that the reduction of in particular microbes occurring at low abundance resulted in an increased aphid body size, as well as an increase in the biomass of *Beta vulgaris* and *Brassica oleracea*. However, until now a more frequently used approach to increase the complexity in studies of microbe-plant-insect interactions has been the use of a combination of several microbial strains. To properly determine the effect of these mixtures, it is required to also evaluate the effect of the individual strains, which is difficult to achieve when applying commercial mixtures of microbes. In any case, no general trend has emerged yet in the effects that an increase of microbial complexity has on the microbe-plant-insect interactions, with evidence showing stronger (Saravanakumar *et al.*, 2007; Currie *et al.*, 2011), weaker (Gange *et al.*, 2003) and no effects (Martinuz *et al.*, 2012) on herbivores aboveground.

One of the factors that can determine the effectiveness of a mixture of microbial strains on plant-mediated effects against herbivores is their genetic relatedness. In a recent study, the effects of four genotypes of the mycorrhizal fungus *R. irregularis*, inoculated alone or in combination, on strawberry plant growth and resistance to the generalist herbivore caterpillar *S. littoralis* were assessed (Roger *et al.*, 2013). Caterpillar fresh weight was reduced by most mycorrhizal treatments, with similar effects of single or dual fungal inoculations. Interestingly, when compared to single inoculation, dual inoculation of genetically very distant isolates affected plant performance parameters stronger than dual inoculation of closely and moderately related isolates. Although in this example herbivore performance was not affected, this could be one of the criteria when searching for powerful combinations of microbes to promote plant growth.

A different factor to consider when combining strains is the change in physiology that the microbial strains induce in the plant. Evidence is accumulating that different strains of root colonizing microbes can mediate ISR via different signaling pathways (Van Oosten *et al.*, 2008; Van Wees *et al.*, 2008; Jung *et al.*, 2012; Van de Mortel *et al.*, 2012). In *A. thaliana*, the strains *P. fluorescens* WCS417r and SS101 decrease the performance of the generalist leaf chewer *Spodoptera exigua* (Van Oosten *et al.*, 2008; Van Wees *et al.*, 2008; Jung *et al.*, 2012; Van de Mortel *et al.*, 2012). Whereas strain WCS417r is known to induce resistance to pathogens via JA- and ET-dependent signaling pathways (Pieterse *et al.*, 1998), strain SS101 acts via the SA-pathway and induction of glucosinolate and

camalexin biosynthesis (Van de Mortel *et al.*, 2012). From these examples, we may speculate that the combined application of root-associated microbes acting via different phytohormonal signaling pathways may enhance plant defense to either pathogens or insect herbivores (Figure 1). Supporting this idea, in cucumber, co-inoculation of non-pathogenic *Trichoderma harzianum* and *Pseudomonas sp*. contributed to a significantly enhanced level of resistance upon challenge by the stem pathogen *Fusarium oxysporum* by activating both JA- and SA-dependent defense responses in comparison to individual treatments (Alizadeh *et al.*, 2013). In accordance, the expression of the defense-associated genes β-*1,3-glucanase*, *CHIT1*, *PR1,* encoding glucanase, chitinase and pathogenesis-related protein respectively, were significantly more pronounced after treatment with a mixture of microbes than with individual strains. Whether activation of both JA- and SA-signaling pathways will also induce the biosynthesis of a higher diversity of secondary metabolites remains to be investigated. Moreover, it can also be hypothesized that some combinations of microbes antagonize each other's effects due to phytohormonal crosstalk within the plant, but to our knowledge no examples of this have been recorded yet. Investigating the interactive effects of different soil community members is important for a thorough understanding of their plant-mediated effects on insect herbivores.

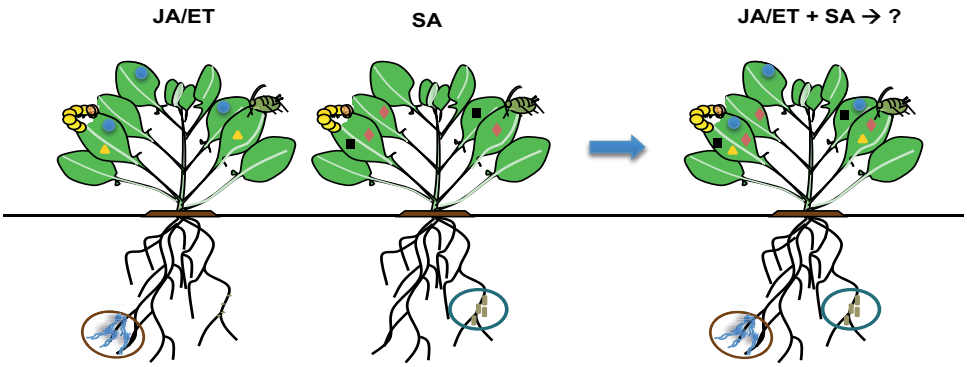

Figure 1. Selected species of root-associated microbes are known to elicit induced systemic resistance (ISR) by priming for enhanced expression of plant defense-associated genes, which become active only after insect or pathogen attack. Depending on microbe species (indicated in brown or blue circles) or strain, ISR can be triggered via JA/ET- or SA-signaling pathways, in which each pathway activates different sets of defense-associated genes. It is hypothesized that application of multiple root-associated microbes that mediate ISR via different signaling pathways may activate higher diversity of defense-associated genes that can enhance plant defense against insects or pathogens. Crosstalk between multiple signaling pathways (JA/ET – SA) regulating ISR within the plant and on how it will affect the outcome of interactions is not known. Different shape of symbols in the leaves represents different defense-associated genes.

PLANT-MEDIATED EFFECTS OF INSECT HERBIVORES ON NON-PATHOGENIC SOIL MICROBES

Upon herbivory, plants respond in several ways that can affect microbe-plant interactions, for instance through the activation of defenses in distal parts, via changes in root exudates or by modifying soil characteristics. Resistance traits induced in certain plant organs and tissues following pathogen or insect attack can be transported to distant tissues and may affect belowground microbes (Doornbos *et al.*, 2011). For instance, in pepper, sap-sucking whiteflies or aphids induce the up-regulation of both SA-dependent and JA-dependent genes not only in leaves but also in roots (Yang *et al.*, 2011b; Lee *et al.*, 2012b). Interestingly, these defense activations did not equally affect all soil microbes. For instance, repeated leaf mechanical wounding of *Medicago truncatula* increased levels of JA locally and systemically leading to enhanced mycorrhizal colonization, whereas colonization by rhizobacteria was not affected (Landgraf *et al.*, 2012).

Moreover, plants can exude/emit compounds belowground to actively recruit specific belowground beneficial organisms. For instance, attack by the foliar pathogen *P. syringae* triggers the secretion of malic acid by *A. thaliana* roots that attract the beneficial rhizobacterium *B. subtilis* (Rudrappa *et al.*, 2008). Foliar infection by the pathogen induced the expression of a malic acid transporter leading to an increased level of malic acid in the rhizosphere (Lakshmanan *et al.*, 2012). Similarly, in maize benzoxazinoids attract *P. putida* (Neal *et al.* 2012). Benzoxazinoids (e.g. DIMBOA) are secondary metabolites that accumulate after herbivory in cereal plants (Erb *et al.*, 2009; Ahmad *et al.*, 2011). Whether they play a role in microbe recruitment after aboveground herbivory remains to be proven. Recently, the first evidence of recruitment of beneficial root microbes after above-ground herbivory has been shown: aphid feeding increased the population of the non-pathogenic rhizobacterium *B. subtilis* GB03 in the rhizosphere of sweet pepper plants (*Capsicum annuum*)(Lee *et al.*, 2012b). However, the chemical cue that triggers the increased colonization has not been discovered yet. This study reveals a new type of interactions and the question arises how multiple herbivory would affect colonization level of root-associated microbes.

Although microbe-plant interactions are established before herbivores will attack those plants, the dynamics of this process are not yet well understood (Heil, 2011). For instance, herbivory may affect via the root exudates certain species of microbes and modify the initial microbiome of a plant. This modified microbiome may have different effects on further herbivore attack on the same plants, or even on the insect interactions

with later successional plants. A study using ragwort plants (*Jacobea vulgaris*) showed that both above- and below-ground herbivory gave specific effects on the composition of the soil fungal community, possibly by changing root exudation. Remarkably, these changes affected interactions of preceding plants with aboveground herbivores and parasitoids, providing evidence that herbivory influences plant-soil feedback responses via changes in the community of soil-borne microbes (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013). Evidence that root herbivory influences root-associated microbes via changes in root exudation was also found in maize. Feeding by western corn rootworm (WCR) larvae changes composition of the microbial community in the rhizosphere, depending on soil type and maize line (Dematheis *et al.*, 2012b). This study indicated that the bacterial community was more affected by the presence of WCR larvae than the fungal community. Interestingly, in all soil types an increased abundance of the phenol-degrading bacterium *Acinetobacter calcoaceticus* was found, which was associated with changes in plant root exudation in response to feeding by WCR larvae. Whether changes in microbial communities affect feeding behaviour of WCR larvae needs further investigation. Using the same system, they also found the presence of rhizosphere microbes in the gut of WCR larvae. The complexity of the community of rhizosphere microbes in the gut was reduced in comparison with that in the rhizosphere, indicating a highly selective condition of the digestive environment (Dematheis *et al.*, 2012a). The biological role of the rhizosphere-associated microbes in the gut of WCR still needs to be unraveled and can potentially be used in new pest control strategies.

PLANT-MEDIATED INTERACTIONS BETWEEN ROOT-ASSOCIATED MICROBES AND INSECTS: THE ROLE OF PLANT HORMONES

Plant hormones function as signal molecules regulating plant growth, development and responses to biotic and abiotic stimuli. The phytohormone JA is a lipid-derived compound playing a prominent role in regulating plant growth and defense against various attackers (Browse, 2005; Wasternack, 2007; Pieterse *et al.*, 2012). JA regulates various aspects of plant growth and development such as seed germination, root growth and flower development (Wasternack, 2007). Moreover, JA functions as the main regulator in the induction of broad-spectrum defense responses to insect herbivores through formation of trichomes as well as enhanced synthesis of proteinase inhibitors (PIs), volatiles, alkaloids, and glucosinolates (Howe and Jander, 2008; Erb *et al.*, 2012). Induction of JA-signaling mainly occurs after attack by necrotrophic pathogens, tissue-chewing insects such as caterpillars, and cell-content feeding insects such as thrips (De Vos *et al.*, 2005).

JA is also responsible for the delivery of long-distance signaling molecules in several plant species (Schilmiller and Howe, 2005; Heil and Ton, 2008; Sogabe *et al.*, 2011; Ankala *et al.*, 2013). Interestingly, JA- signaling has also been described as the main pathway in ISR against aboveground herbivores and is stimulated by root-associated microbes (Van Oosten *et al.*, 2008; Pineda *et al.*, 2010; Jung *et al.*, 2012). The activation of the JA signaling pathway also affects the plant's interaction with root-colonizing microbes, for instance by altering the composition of root-associated bacterial communities (Carvalhais *et al.*, 2013). Herbivory by *Pieris rapae* and *Helicoverpa armigera* caterpillars activated a branch of the JA-signaling pathway that is regulated by the transcription factor MYC2 (De Vos *et al.*, 2005; Dombrecht *et al.*, 2007; Verhage *et al.*, 2011). MYC2 positively regulates the biosynthesis of flavonoids (Dombrecht *et al.*, 2007), widely distributed plant secondary metabolites that often function as feeding deterrents to herbivores and as pigments attracting pollinators (Schoonhoven *et al.*, 2005). Interestingly, flavonoids are also present in root exudates and are crucial in the establishment of rhizobacterial colonization (Ferguson and Mathesius, 2003; Steinkellner *et al.*, 2007; Dennis *et al.*, 2010; Zamioudis and Pieterse, 2012). It is known that exogenous application of the volatile JA-derivative methyl jasmonate (MeJA) increases the release of flavonoids from plant roots (Faure, 2009; Buer *et al.*, 2010). Whether JA-induced synthesis of flavonoids is involved in active recruitment or changes of non-pathogenic soil-borne microbe populations following herbivory remains to be investigated.

In addition to JA, SA is another key hormone regulating plant defense against biotrophic pathogens and against insect herbivores with a piercing-sucking feeding mode, such as aphids and whiteflies (Mewis *et al.*, 2005; Pieterse and Dicke, 2007; Zarate *et al.*, 2007; Kusnierczyk *et al.*, 2008; Wu, 2010). More recent findings also suggest a role of SA-dependent signaling in the plant response to insect herbivore oviposition (Browse, 2009; Reymond, 2013). To activate a defense response, SA signaling is transduced via the regulatory protein Non-expressor of Pathogenesis-Related genes1 (NPR1), which functions as transcriptional co-activator of SA-responsive genes such as pathogenesis-related proteins (*PR*) (Dong, 2004). NPR1 is required to mount ISR against pathogens by different beneficial microbes, independently of the pathways that mediate the ISR (Pieterse *et al.*, 1998; Segarra *et al.*, 2009; Van de Mortel *et al.*, 2012) and it also functions as an important node modulating SA- and JA-signaling crosstalk (Spoel *et al.*, 2003; Pieterse and Van Loon, 2004; Pieterse *et al.*, 2012). Concurrently, SA-dependent signaling is crucial in interactions of plant roots with non-pathogenic microbes. It has been suggested that in the initial stage of symbiosis, non-pathogenic microbes are sensitive to

SA-regulated defense responses (Zamioudis and Pieterse, 2012). SA-signaling has been reported to negatively affect rhizobial, mycorrhizal and rhizobacterial colonization (Van Spronsen *et al.*, 2003; Doornbos *et al.*, 2011). In plant-rhizobia interactions, transient overexpression of *NPR1* in *M. truncatula* suppressed symbiosis, whereas inhibition of *NPR1* induces the acceleration of *Sinorhizobium meliloti* symbiosis (Peleg-Grossman *et al.*, 2009). This suggests that initially the plant recognizes non-pathogenic microbes as alien organisms and, therefore, activates defense mechanisms via SA-dependent signaling pathways (Zamioudis and Pieterse, 2012). In the context of multiple herbivore attack, how crosstalk between signaling pathways induced by insects with different feeding characteristics will affect the level of colonization by root-associated microbes is an area for future investigation.

The JA-signaling pathway also cross-communicates with the ET and ABA signaling pathways through the use of common transcription factors. In *A. thaliana*, the JA-pathway has two main branches, the MYC2- and ERF-branches, each activating different sets of JA-responsive genes (Lorenzo and Solano, 2005; Wasternack, 2007; Pieterse *et al.*, 2012; Kazan and Manners, 2013). The MYC2-branch acts in synergy with ABA-signaling, whereas the ERF branch cross-communicates with the ET-signaling pathway (Abe *et al.*, 2002; Lorenzo *et al.*, 2003). Herbivory by *P. rapae* and *Helicoverpa armigera* caterpillars activates the branch that is regulated by the transcription factor MYC2 and enhances the expression of Vegetative Storage Protein 2 (*VSP2)* (De Vos *et al.*, 2005; Dombrecht *et al.*, 2007; Verhage *et al.*, 2011), which is an acid phosphatase having anti-insect activity (Liu *et al.*, 2005). The transcription factor MYC2 is also required to mount ISR against pathogens (Pozo *et al.*, 2008). Recent evidence showed the importance of ABA and ET signaling also in the colonization of plants by non-pathogenic microbes (Camehl *et al.*, 2010; Martin-Rodriguez *et al.*, 2011). In *Arabidopsis*, overexpression of ERF1 had a strong negative effect on root colonization by the beneficial fungus *Piriformospora indica* (Camehl *et al.*, 2010). This study suggested that ET-signaling and ET-targeted transcription factors are crucial to balance beneficial and non-beneficial traits in the symbiosis. In tomato, a functional ABA-signaling pathway was demonstrated to be required for mycorrhization (Martin-Rodriguez *et al.*, 2011). Moreover, there is also negative crosstalk between the ABA- and ET-signaling pathways, in which ABA deficiency enhances the ET level and negatively regulates colonization by mycorrhizae. However, how crosstalk between JA-ABA, JA-ET and ABA-ET will affect microbe-plant-insect interactions remains to be elucidated.

POTENTIAL ROLE OF NEW HORMONAL PLAYERS IN REGULATING MICROBE - PLANT - INSECT INTERACTIONS

Increasing evidence shows that the final outcome of plant defense against various attackers is also depending on hormones other than JA and SA (Robert-Seilaniantz *et al.*, 2011). Attention is now shifting to explore plant hormones such as auxin, CK, GA, brassinosteroid (BR) and strigolactone (SL), all of them important in many aspects of plant growth and development (Ohnishi *et al.*, 2006; Sakakibara, 2006; Giron *et al.*, 2013; Liu *et al.*, 2013). For instance, in addition to controlling plant growth via degradation of growth-repressing DELLA proteins, GAs have been indicated to enhance SA-signaling and to increase resistance to biotrophic pathogens (Navarro *et al.*, 2008). Although information on the effect on insect herbivores is scarce (Yang *et al.*, 2011a), the fact that several of these hormones can modulate JA- and SA-signaling (Campos *et al.*, 2009; Ballaré, 2011) suggests that they are also involved in defense responses to herbivores. Interestingly, these hormones are also involved in regulating plant interactions with non-pathogenic microbes. For instance, GA positively regulates nodulation by rhizobia (Ryu *et al.*, 2012), reduced CK levels seem to stimulate mycorrhizal hyphal growth in the roots (Cosme and Wurst, 2013), and SL induces hyphal branching and further establishment of mycorrhizal symbioses (Liu *et al.*, 2013).

Recent experimental evidence suggests that non-pathogenic microbes are able to modify plant hormone metabolism to increase plant growth capacity. In *A. thaliana*, auxin-, BR-, GA-, SA- and ET-signal transduction pathways are involved in elicitation of growth promotion by several species of non-pathogenic microbes (Ryu *et al.*, 2005; Contreras-Cornejo *et al.*, 2009; Zamioudis *et al.*, 2013). Auxin signalling, known to be critical in regulating plant growth and development, seems to be involved in the effects that non-pathogenic microbes have on root architecture and plant growth (Contreras-Cornejo *et al.*, 2009; Zamioudis *et al.*, 2013). For instance, growth promotion and root development induced by *Trichoderma virens* is reduced in *Arabidopsis*-mutants, *aux1*, *eir1-1* and *axr1-3,* impaired in auxin-signaling (Contreras-Cornejo *et al.*, 2009). Several species of non-pathogenic root-associated microbes are known to induce higher auxin concentration *in planta* (Dodd *et al.*, 2010), whereas in response to herbivory, endogenous auxin concentration varies depending on insect feeding mode (Tooker and De Moraes, 2011; Soler *et al.*, 2013). Similarly, intact CK-signaling is responsible for plant-growth promotion by *B. megaterium* in *A. thaliana* (Ortíz-Castro *et al.*, 2008). In lettuce, increased CK content in roots and shoots was observed following colonization of roots by *Bacillus subtilis* (Arkhipova *et al.*, 2005). In plant-insect interactions, CK-

related transcripts are strongly upregulated following treatment with fatty acid-amino acid conjugates (FACs), that are insect-derived elicitors (Erb *et al.*, 2012). A very interesting aspect of these hormones for below-aboveground interactions is their role as long-distance signaling molecules (Soler *et al.*, 2013). Auxin has a role in communicating nitrogen shortage between shoot and root (Tamaki and Mercier, 2007). In contrast, CK has been proposed as negative regulator of nitrogen-uptake related genes, which means that CK is produced if an adequate nitrogen level is present, possibly to inhibit nitrogen uptake in the roots (Sakakibara, 2006; Kudo *et al.*, 2010; Kiba *et al.*, 2011). However, how possible crosstalk between JA, SA, and these new hormonal players affects interactions involving microbes, plants and insects is not known yet.

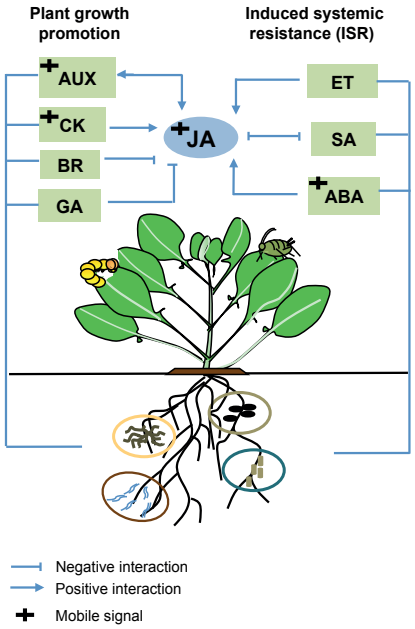

Figure 2. Model of interactions between plant hormones regulating plant defense and development in microbe-plant-insect interactions. Different root-associated microbes elicit induced systemic resistance (ISR) via jasmonic acid- (JA), ethylene- (ET,) salicylic acid- (SA) or abscisic acid- (ABA) signaling pathways. Root-associated microbes are also known to induce plant growth promotion via auxin- (AUX), cytokinin- (CK), brassinosteroid- (BR) and gibberellin- (GA) signaling pathways. JA is considered the main hormone regulating the switch from growth to defense through positive and negative crosstalk with other plant hormones. Since root-associated microbes enhance plant defense and growth, these microbes may benefit plant fitness by relieving the trade-off between growth and defense. Investigating the regulatory mechanisms of crosstalk between defense signaling pathways (JA, SA, ET) and growth signaling pathways (AUX, CK, BR, GA) in this system may unveil how plants regulate their resources to invest in growth and defense in the presence of root-associated microbes.

Plants need to regulate resources in the most efficient way to optimally invest in growth and defense. Recent discoveries in plant genomics have shown that hormone signaling networks involved in growth and defense are interconnected, allowing plants to invest in growth under suitable conditions or in defense when they sense attacker-derived signals (Pieterse *et al.*, 2012; Kazan and Manners, 2013). JA has been indicated as the core phytohormone mediating the switch from growth to defense via its positive and antagonistic crosstalk with other plant hormones, such as auxin, GA and CK (Wasternack, 2007; Pauwels *et al.*, 2009; Ballaré, 2011; Yang *et al.*, 2012; Hou *et al.*, 2013; Kazan and Manners, 2013). In parallel, root-associated microbes are known to increase plant defense and promote plant growth. It is hypothesized that root-inhabiting beneficial microbes can benefit plant fitness by relieving the trade-off between growth and defense (Bennett *et al.*, 2006). However, knowledge on how plants differentially regulate their resources to invest in growth and defense in the presence of beneficial root-inhabiting microbes is not available. Because there is an overlap in how new hormonal players regulate plant defense to insect herbivory and how root-associated microbes promote plant growth, unveiling the regulatory mechanisms of crosstalk between defense signaling pathways (JA, SA, ET) and growth signaling pathways (auxin, GA, CK) and how this will affect the trade-off between growth and defense will be fruitful areas of further investigation (Figure 2).

FUTURE PERSPECTIVES

Over the last two decades, multiple studies in different ecological settings have shown that in nature root-associated microbes can affect insects aboveground. However, the underlying mechanisms of microbe-plant-insect interactions have only recently started to be understood. Using simplified systems with one species of microbe and one species of herbivore, experimental evidence has shown that selected species of root-colonizing microbes may augment plant defense by priming for enhanced expression of defense-associated genes regulated by either JA/ET- or SA-signaling pathways. However, how complex communities of root-inhabiting microbes differentially modulate plant defense and how this will affect herbivores above- and below-ground is a challenging area of future studies. Examples given in this review demonstrate that the application of multiple root-associated microbes can have neutral or even positive effects on the performance of insect herbivores. The fact that in realistic field situations, the positive effect on herbivores could revert to negative effects through increased indirect plant defense by increased attraction of natural enemies indicates the significance of an holistic approach in the study of microbe-plant-insect interactions. Major issues are to

gain mechanistic insight in how crosstalk between different microbe-activated signaling pathways affects the level of plant resistance to various insects and to extend studies to natural conditions to assess its ecological implications. Moreover, in response to attack by multiple insect herbivores, plants also activate different hormone-mediated signaling pathways depending on feeding characteristics of the insects and crosstalk between these pathways can have consequences on interactions of plants with root-associated microbes. To our knowledge, no study has addressed how for instance JA-SA crosstalk induced by multiple herbivores would affect the level of mutualistic interactions between plants and root-associated microbes.

Plants have several layers of defense mechanisms to withstand insect attack. In addition to plant indirect defense by attraction of the herbivore's natural enemies following herbivory, a growing body of evidence shows that to strengthen their layers of defense, plants can actively recruit help from below-ground organisms following attack by foliar pathogens. However, experimental evidence showing that plants develop similar mechanisms following insect herbivory is lacking. If similar mechanisms are uncovered, our understanding of plant defense will grow. Apart from HIPVs emitted by plants, numerous root-associated microbes are also known to produce volatile organic compounds (VOCs) that could affect insects directly but it is unknown if microbe-derived VOCs directly influence plant-associated insect communities. The review by (Kupferschmied *et al.*, 2013) in this issue provides valuable information on how various traits of root-associated *Pseudomonas* can have direct effects on below-ground pest insects. This hold promise for broader application of root-associated microbes in pest control above- and below-ground.

In their struggle to survive, plants face the dilemma of allocating resources to growth or defense. It is hypothesized that support from root-inhabiting microbes may relieve plants from this trade-off by increasing their access to nutrients (Bennett *et al.*, 2006). However, how plants differentially regulate their resources in the presence of root-associated microbes and which regulatory mechanisms are involved (i.e. hormones, transcription factors) and how these will affect plant interactions with insects still need to be investigated. For instance, crosstalk between JA and SA and between JA and ET in signaling networks is known to be important in the regulation of plant defense against pathogens and insect herbivores. In addition, crosstalk between JA and auxin, JA and GA and JA and CK is thought to play a role in the trade-off between growth and defense. Therefore, it would be interesting to study crosstalk between defense signaling pathways (JA, SA, ET) and growth signaling pathways (auxin, GA, CK) in the context of

microbe-plant-insect interactions. Interestingly, several plant hormones that are known to mediate microbe-induced plant growth promotion such as auxin and CK have also recently been identified as mobile signals connecting shoot and roots (Sakakibara, 2006; Tamaki and Mercier, 2007; Kudo *et al.*, 2010). The role of these mobile signals in microbe-plant-insect interactions would be a promising area of further studies.

Beneficial root-associated microbes have a vast potential as environmentally safe pest control agents above- and below-ground. Application of certain species or strains of non-pathogenic bacterial/fungal species into agricultural soils in order to stimulate plant growth or as biocontrol agent against plant pathogens or insects has been performed for years. In spite of several success stories in the application of these non-pathogenic microbes to promote plant health and growth, inconsistencies have often been reported. One of key factors responsible for the failures is the fast decline in the number of microbial populations being introduced, as reviewed in (Van Veen *et al.*, 1997). The importance of factors such as physiological traits of the microbial agents affecting their competitiveness and survival in the rhizosphere has not been studied in any detail. Interestingly, experimental evidence has shown that specificity of interactions between plant species and associated rhizobacterial communities exist (Smalla *et al.*, 2001; Garbeva *et al.*, 2004; Sugiyama *et al.*, 2013). For decades, application in IPM of microbes from the genera *Pseudomonas*, *Bacillus* and *Trichoderma*, known to colonize many plants from different families, has been common practice. However, the fact that there is a certain level of specificity in the interactions between plant species and their root-associated microbes may indicate that application of certain microbial genera to non-host plants can affect their survival in the rhizosphere. Therefore, we should start identifying plant family-specific groups of root-inhabiting microbes and apply them to their proper host plants to increase their survival in the rhizosphere. Identifying microbial strains from extreme environments, such as insect/disease suppressive soils or the rhizosphere of plants that produce high toxin levels may be a way of obtaining highly competitive microbes. Following the isolation, the characteristics of the isolated microbes in triggering ISR, stimulating plant growth, and competitiveness in the rhizosphere should be evaluated. For a community approach, a thorough selection procedure combining several species of microbes, that are genetically distant and that mediate ISR via different pathways or with different microbe-associated molecular pattern (MAMPs) may enhance competitiveness of the microbes in the rhizosphere, and the induction of ISR. Moreover, understanding the mechanisms and ecology of indirect and direct plant-mediated mechanisms operating between communities of root-associated microbes and

insect communities above- and below-ground can increase the reliability and durability of application of beneficial microbes in IPM.

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3

Variation in plant-mediated interactions between rhizobacteria and caterpillars: potential role of soil composition

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ABSTRACT

Selected strains of non-pathogenic rhizobacteria can trigger induced systemic resistance (ISR) in plants against aboveground insect herbivores. However, the underlying mechanisms of plant-mediated interactions between rhizobacteria and herbivorous insects are still poorly understood. Using *Arabidopsis thaliana* Col-0 - *Pseudomonas fluorescens* WCS417r as a model system, we investigated the performance and the molecular mechanisms underlying plant-mediated effects of rhizobacteria on the generalist caterpillar *Mamestra brassicae* and the specialist *Pieris brassicae*. Rhizobacteria colonization of *Arabidopsis* roots resulted in decreased larval weight of *M. brassicae*, whereas no effect was observed on larval weight of *P. brassicae*. Using a jasmonic acid (JA)-impaired mutant (*dde2-2*), we confirmed the importance of JA in rhizobacteria-mediated ISR against *M. brassicae*. Interestingly, in some experiments we also observed rhizobacteria-induced systemic susceptibility to *M. brassicae*. The role of soil composition in the variable outcomes of microbe-plant-insect interactions was then assessed by comparing *M. brassicae* performance and gene transcription in plants grown in potting soil or a mixture of potting soil and sand in a 1:1 ratio. In a mixture of potting soil and sand, rhizobacteria treatment had a consistent negative effect on *M. brassicae*, whereas the effect was more variable in potting soil. Interestingly, at 24 hpi rhizobacteria treatment primed plants grown in a mixture of potting soil and sand for a stronger expression of the JA- and ethylene-regulated genes *PDF1.2* and *HEL*. Our study shows that soil composition can modulate rhizobacteria-plant-insect interactions, and is a factor that should be considered when studying these belowground–aboveground interactions.

Keywords: *Arabidopsis thaliana*, induced systemic resistance, jasmonic acid, *Mamestra brassicae*, *Pieris brassicae*, priming, *Pseudomonas fluorescens*, rhizobacteria.

INTRODUCTION

In natural and agricultural ecosystems, plants are members of complex communities and interact with a broad spectrum of organisms. In addition to interactions with plant enemies such as insect herbivores or microbial pathogens, plants frequently interact with belowground non-pathogenic microbes such as mycorrhizae and rhizobacteria. The associations between plants and mycorrhizae and between plants and rhizobacteria are generally mutualistic, positively affecting plant fitness (Pieterse and Dicke, 2007; Van der Heijden *et al.*, 2008; Schwachtje *et al.*, 2011). For instance, several species of non-pathogenic microbes i.e. in the genera *Pseudomonas, Bacillus*, *Burkholderia* provide a benefit to plants by increasing several plant growth parameters (Barea *et al.*, 2005; Poupin *et al.*, 2013; Zamioudis *et al.*, 2013). Moreover, the root-associated microbes may also trigger molecular and physiological changes in the plants mediating enhanced plant defense to harmful organisms, referred to as induced systemic resistance (ISR) (Van Oosten *et al.*, 2008; De Vleesschauwer *et al.*, 2009; Jung *et al.*, 2012; Pangesti *et al.*, 2013). Most studies investigated how root-associated microbes affect plant resistance to various types of plant pathogens (Van Loon *et al.*, 1998; De Vleesschauwer *et al.*, 2009), and only in the last two decades studies exploring the potential use of these non-pathogenic microbes in plant protection to insect herbivores have been conducted (Bennett *et al.*, 2009; Pineda *et al.*, 2010). In spite of experimental evidence showing the promising role of non-pathogenic root-associated microbes as crop protection agents (Van Oosten *et al.*, 2008; Pineda *et al.*, 2010; Song *et al.*, 2013), recent evidence shows that these microbes do not always provide protective functions to the plants and can also trigger induced susceptibility to insect herbivores (Pineda *et al.*, 2012; Roger *et al.*, 2013; Shavit *et al.*, 2013; D'Alessandro *et al.*, 2014).

Only limited knowledge is available on the factors that contribute to such contrasting effects in the outcome of plant-mediated interactions between root-associated microbes and shoot-feeding insects and several studies have proposed that the outcome is context-dependent (Gange *et al.*, 2005; Hartley and Gange, 2009; Koricheva *et al.*, 2009). Biotic factors such as identity of the host plant, root-associated microbes and insect species have been proposed to cause variation in the outcome of microbe-plant-insect interactions (Hartley and Gange, 2009). Modification in plant chemistry is usually at the basis of such multitrophic interactions; for example, several root-associated microbe-mediated changes in transcriptomic and metabolic profiles of host plants are associated with increased plant defenses to insect herbivores (Van Oosten *et al.*, 2008; Van de Mortel *et al.*, 2012). Usually, generalist insects are more sensitive to increased concentrations

of plant defensive compounds than specialists (Schoonhoven *et al.*, 2005). Considering the degree of specialisation and feeding mode of the insects, a general pattern of plant-mediated effects of root-associated microbes on insect herbivores has been proposed, i.e. a negative effect on generalist chewing insects and mesophyll feeders and a positive or neutral effect on specialist chewing insects and phloem feeders (Pineda *et al.*, 2010). Additionally, these belowground microbes are also shown to enhance plant nutritional status, for instance several arbuscular mycorrhizal (AM) fungi increase nitrogen content of host plants and positively affect the performance of insect herbivores (Gange *et al.*, 2005). Therefore, it has been suggested that the net effect of plant-mediated interactions with non-pathogenic root-associated microbes on the performance of insect herbivores depends on the balance between the negative effects of induced resistance and the positive effects of enhanced plant nutritional quality (Pineda *et al.*, 2010). Interestingly, recent ecological studies show that abiotic factors, such as soil nutrients or water stress can alter the strength of mutualistic associations between plants and root-associated microbes (Compant *et al.*, 2010; Hoeksema *et al.*, 2010; Balzergue *et al.*, 2011). However, along which mechanisms abiotic factors influence microbe-plant-insect interactions still remains to be unravelled.

To develop defense mechanisms against insect attackers and simultaneously establish associations with non-pathogenic soil microbes, plants use hormones (e.g. jasmonic acid (JA), ethylene (ET), salicylic acid (SA), abscisic acid (ABA)) as signal molecules to coordinate their immune responses (Pieterse *et al.*, 2012; Zamioudis and Pieterse, 2012). In plant defense against insects, the phytohormones JA, ET, and SA regulate the activation of different signaling pathways that are known to be crucial in the regulation of plant defenses against specific types of attackers (Pieterse and Dicke, 2007; Erb *et al.*, 2012; Pieterse *et al.*, 2012; Stam *et al.*, 2014). For instance, chewing insects and necrotrophic pathogens are generally more sensitive to JA- and ET-dependent defenses, whereas piercing-sucking insects and biotrophic pathogens are generally more affected by SA-dependent defenses (Howe and Jander, 2008; Pieterse *et al.*, 2012). Remarkably, the phytohormones JA, ET, and SA also underlie interactions between plants and non-pathogenic rhizobacteria as well as plant-mediated effects of these rhizobacteria on herbivores (Pieterse *et al.*, 2012; Zamioudis and Pieterse, 2012). Apart from a few exceptions (Barriuso *et al.*, 2008; Van de Mortel *et al.*, 2012), non-pathogenic rhizobacteria-mediated ISR functions independent of SA, but depends on an intact plant response to JA and ET (Pieterse *et al.*, 2002; Ahn *et al.*, 2007; Weller *et al.*, 2012). For example, the well-studied *Pseudomonas fluorescens* strain WCS417r triggers ISR via

JA/ET signaling pathways and the plants are primed for a stronger or faster expression of defense responses that become active only after pathogen or insect attack (Pieterse *et al.*, 2002; Van Oosten *et al.*, 2008; Van der Ent *et al.*, 2009). Multiple studies with mutants have confirmed the role of JA/ET in ISR against pathogens (Pieterse *et al.*, 2012), but such evidence in relation to insects is still missing.

The importance of linking ecological and molecular mechanisms in the context of microbe-plant-insect interactions has received increased attention, but still a big challenge is to identify the factors that can help us to predict patterns in these interactions. Using the rhizobacteria *P. fluorescens* WCS417r-*Arabidopsis thaliana* Col-0 model system, the present study investigates: 1) how rhizobacteria-mediated ISR affects the generalist *M. brassicae* caterpillar and the specialist *P. brassicae* caterpillar; 2) the mechanism involved in these multitrophic interactions, 3) the effect of soil composition on microbe-plant-insect interactions. Based on knowledge from previous studies on these multitrophic interactions, we formulated the following hypotheses. Our first hypothesis is that *P. fluorescens* WCS417r-mediated ISR will reduce the performance of the generalist herbivore *M. brassicae* and will have no effect on the performance of the specialist *P. brassicae*. Our second hypothesis is that *P. fluorescens* WCS417r treatment leads to an enhanced induction by insect attack of key genes in the JA/ET pathways such as *Lipoxygenase 2* (*LOX2*) and *Plant Defensin 1.2* (*PDF1.2*). Furthermore, *P. fluorescens* WCS417r-mediated ISR against *M. brassicae* is expected to be eliminated in a mutant defective in the JA-signaling pathway, whereas *P. fluorescens* WCS417r-treatment is expected to have no effect on the performance of *P. brassicae* in both wild type and mutant plants. Our third hypothesis is that the variation that is present in the plant-mediated effect of *P. fluorescens* WCS417r on herbivores can be partially explained by soil composition which will be studied by comparing the effect in potting soil with the effect in potting soil that is diluted with sand. We expect that in the mixture of soil and sand the interaction between *P. fluorescens* WCS417r and plant is stronger and results in a more consistent effect on *M. brassicae* performance and also stronger expression of defense-associated genes.

MATERIALS & METHODS

Plants and insects

Seeds of *Arabidopsis thaliana* Col-0 and the male-sterile mutant *dde2-2* (*delayed-dehiscence2-2*) were sown in commercial potting soil for *Arabidopsis* (Lentse Arabidopsis-

grond, Lent, The Netherlands). Seedlings (10-days old) were transplanted into pots (120 ml) containing potting soil. Prior to the transplant, a suspension of *P. fluorescens* WCS417r was added to the soil (50 ml of rhizobacteria suspension per kg of soil) and mixed carefully to a final density of 5 x 10^7 cfu g^{-1} soil, whereas an equal amount of 10 mM $MgSO_4$ was added to the soil for the control treatment. Plants were cultivated in a growth chamber under 8:16 h photo:scotophase (200 µmol m^{-2} s^{-1}; TL-D36W/840, Philips, Eindhoven, the Netherlands) at 21 ± 1 °C and 60 ± 10 % relative humidity (RH). Plants with control and *P. fluorescens* WCS417r treatments were kept in different trays to avoid transmission of the rhizobacteria and tray positions were randomized three times a week to prevent spatial effects. The plants were watered three times a week, adding a total volume of 50 ml water for each pot per week. All soil used was autoclaved twice at 121 °C for 20 min with a 24 h interval. In all experiments, 5-6 week old plants in the vegetative stage were used.

The generalist insect herbivore *Mamestra brassicae* L. (Lepidoptera: Noctuidae; Cabbage moth) and the specialist *Pieris brassicae* L. (Lepidoptera: Pieridae; Large Cabbage White butterfly) were reared on *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) in a climate chamber (22 ± 2 °C, 40 - 50 % RH, 16:8 h photo:scotophase). Newly-emerged larvae were used in the experiments.

Rhizobacteria growth conditions, inoculation to soil media and quantification

A rifampicin-resistant, non-pathogenic rhizobacterium strain *Pseudomonas fluorescens* WCS417r (abbreviated as *Pf.* WCS417r) was used in this study. The strain was isolated from the rhizosphere of wheat grown in soil suppressive against take-all disease caused by *Gaeumannomyces graminis pv. tritici* (Poupin *et al.*, 2013). *Pf.* WCS417r was grown on King's B (KB) medium agar plates (Pieterse *et al.*, 1996) containing rifampicin (25 µg ml^{-1}) for 48 h at 28 °C. Prior to mixing with autoclaved soil, bacterial cells were collected, resuspended in 10 mM $MgSO_4$ and adjusted to a cell density of 1 x 10^9 cfu ml^{-1} (OD660 = 1.0). Rhizobacterial treatment of plants for experiments 1, 2, and 3: 50 ml of the bacterial suspension was mixed per kg of autoclaved potting soil; for control treatment, 50 ml of 10 mM $MgSO_4$ was mixed per kg of autoclaved potting soil. For experiment 4, in order to inoculate bacterial cells in an equal density to 100 % potting soil (P) and to a mixture of potting soil and sand (1P:1S), we used the specific weight of P soil as a reference to calculate how much rhizobacterial culture is needed for P and 1P:1S soils.

Colonization of *A. thaliana* roots by *Pf.* WCS417r was quantified as described for each bioassay to confirm that the colonization met the required threshold for ISR of 10^5 cfu.g^{-1} root (Raaijmakers *et al.*, 1995). Roots were harvested, weighed and shaken vigorously for 1 min in 10 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (425-600 µm, Sigma-Aldrich, St. Louis, Missouri, USA). Proper dilutions were plated onto KB agar medium supplemented with cycloheximide (100 µg ml^{-1}), ampicillin (50 µg ml^{-1}), chloramphenicol (13 µg ml^{-1}), and rifampicin (150 µg ml^{-1}), a combination selective for rifampicin-resistant fluorescent *Pseudomonas* spp. (Pieterse *et al.*, 1998) (Geels, 1983). The dilution plates were incubated for 48 h at 28 °C, after which the number of cfu per gram root fresh weight was determined.

Experiment 1: Effect of rhizobacteria on insects feeding on wild-type plants grown in potting soil

To test the hypothesis that *Pf.* WCS417r has a differential effect on generalist and specialist caterpillars, *Arabidopsis* wild type Col-0 plants were grown in sterile potting soil that was either treated with rhizobacteria or kept untreated. Three newly-emerged larvae of *M. brassicae* or *P. brassicae* were transferred to each *A. thaliana* Col-0 plant using a fine paint brush (N = 20 plant replicates). All plants were then confined in a plastic container (height 14 cm; upper diameter 11 cm, lower diameter 8.5 cm), covered with insect-proof mesh cloth and sealed with elastic bands. On a microbalance (CP2P, Sartorius AG, Göttingen, Germany), *M. brassicae* larvae were weighed to the nearest 0.001 mg at 6- and 12-days post infestation (dpi) and *P. brassicae* larvae were weighed at 6 and 10 dpi. During the insect performance assays, well before the first infested plant was fully consumed by caterpillars, the caterpillars were gently transferred to a second plant. Bioassays were performed in a growth chamber under 16:8 h photo:scotophase (200 µmol m^{-2} s^{-1}) at 21 ± 1 °C and 60 - 70% RH.

Experiment 2: Role of the JA signaling pathway in the interaction between rhizobacteria and herbivores using the *A. thaliana* mutant *dde2-2*

To test the hypothesis that microbe-plant-insect interactions are regulated by the JA signaling pathway, we evaluated the performance of *M. brassicae* and *P. rapae* when feeding on *dde2-2* plants. The *dde2-2* mutant is defective in the *Allene Oxide Synthase* gene (*AOS*), encoding one of the enzymes in the jasmonic acid (JA) biosynthesis pathway (Von Malek *et al.*, 2002). This mutant has the Col-0 background, therefore herbivore

performance was also simultaneously evaluated on Col-0 plants. This experiment was performed as described for Experiment 1, and 20 plant replicates were used per treatment.

Experiment 3: Expression of defense-related genes in plants grown in potting soil

To evaluate the mechanisms involved in rhizobacteria-induced ISR against *M. brassicae* and *P. brassicae*, the expression levels of several genes involved in anti-herbivore defenses were evaluated from Col-0 plants growing in potting soil. The following treatments were arranged: 1) control (**C**): plants without *Pf.* WCS417r, uninfested; 2) rhizobacteria (**R**): plants treated with *Pf.* WCS417r, uninfested; 3) control + *M. brassicae*/*P. brassicae* (**CM/CP**): plants without *Pf.* WCS417r, infested with either *M. brassicae* or *P. brassicae* caterpillars; 4) rhizobacteria + *M. brassicae*/*P. brassicae* (**RM/RP**): plants treated with *Pf.* WCS417r, infested with either *M. brassicae* or *P. brassicae* caterpillars. In insect infestation treatments, three first instar larvae (L1) of *M. brassicae* or *P. brassicae* were transferred to fully expanded leaves as in the performance experiment. Prior to harvesting, caterpillars were gently removed from the infested plants and leaves damaged by feeding larvae (local leaves) were used for gene transcript analyses. Fully expanded leaves of all treatments were harvested at 24 and 72 h after insect infestation (hpi). Leaves of uninfested plants were treated and harvested at similar time points as those of infested plants. For each treatment, five biological replicates were used, each consisting of six to nine local leaves pooled from three individual plants (Pineda *et al.*, 2012). Individual plants of each treatment were confined and covered with insect-proof mesh cloth as previously described in the section about insect performance. Leaf samples were immediately frozen in liquid nitrogen and stored at -80°C for further RNA extraction.

RNA extraction and cDNA synthesis. Leaf samples were ground in liquid nitrogen and total RNA was extracted and purified following the protocol of RNeasy plant mini kit (Qiagen, Hilden, Germany). Subsequently, RNA was eluted in 30 µl RNase free water. DNA contamination was removed by DNase treatment using an RNase-free DNase set (Qiagen, Hilden, Germany). RNA concentration and purification were measured using a NanoDrop ND-100 spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). Ratio of optical density $(OD)_{260/280}$ ~ 2.0 was applied as an assessment of RNA purity. RNA integrity was checked by a chip-based nucleic acid analysis system (Bioanalyzer, Agilent RNA 6000 Nano Kit, Waldbronn, Germany). Subsequently,

samples of RNA (1 μg) were transformed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR analysis. Transcripts of the JA-regulated gene *LIPOXYGENASE 2* (*LOX2*) (At3g45140) and of the JA/ET-regulated gene *PLANT DEFENSIN 1.2* (*PDF1.2*) (At5g44420) were quantified. Additionally, transcript levels of other JA- and JA/ET-regulated genes, i.e. transcription factor *MYC2*/*JASMONATE INSENSITIVE1* (At1g32640), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) (At5g24770), *ABSCISIC ACID 1* (*ABA1*) (At5g67030, *ETHYLENE RESPONSE FACTOR 1* (*ERF1*) (At1g27730) were also assessed (see Suppl. Material). Sequences of gene-specific primers for qRT-PCR are listed in table S1. Efficiency of each primer was determined before qRT-PCR analysis (Pineda *et al.*, 2012). Thermal cycling conditions consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 45 s. For each primer pair, controls without addition of template were performed to confirm that primer dimers were not interfering with detection of amplification. The transcript level for each tested gene was calculated relative to the reference genes *ELONGATION FACTOR 1*α (*EF1*α) (AT5G60390) and *F-BOX FAMILY PROTEIN* (*FBOX*) (AT5G15710) (Remans *et al.*, 2008) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Experiment 4: Influence of soil composition on the plant-mediated interaction between rhizobacteria and *M. brassicae*

To test the hypothesis that soil composition affects the induction of ISR, we evaluated the effect of *Pf.* WCS417r colonization on the performance of *M. brassicae* using 100% potting soil (P) and a 1:1 (v/v) mixture of potting soil : sand (1P:1S). For this experiment, *A. thaliana* Col-0 seeds were sown in river sand (Masonry sand, Van Leusden B.V., The Netherlands). Seedlings (10-day-old) were transplanted into pots (120 ml) containing either P or 1P:1S. Inoculation of rhizobacteria in both soil composition was performed as described above in section "Rhizobacteria growth conditions, inoculation to soil media and quantification". Once a week, 10 ml of half-strength Hoagland solution/pot was added (Van Oosten *et al.*, 2008). *M. brassicae* performance was evaluated as in Experiment 1, except that larval weight was determined at 6 dpi. This experiment was repeated in two independent trials, each with 25 plant replicates per treatment. During the two independent trials, dry weight of total shoot biomass (60 °C, 10 d) was measured at the end of the *M. brassicae* performance experiment, with 10 plant replicates per treatment.

In order to link the effect of *Pf.* WCS417r colonization on the performance of *M. brassicae* with induction of defense gene transcription on *A. thaliana* grown in different soil compositions, we simultaneously performed comparative gene transcription analysis on plants from the same batch as used in the second trial of the herbivore performance. For this experiment, transcript levels of JA- and JA/ET-regulated genes *LOX2*, *PDF1.2* and *HEVEIN-LIKE PROTEIN* (*HEL*) (At3g04720) were assessed. Transcript levels of the JA-regulated genes *LOX2*, *PDF1.2*, *HEL* were quantified comparing treatments C, R, CM, RM in *A. thaliana* Col-0 plants grown in different soil compositions (P vs 1P:1S). We used the same methodology as described for Experiment 2.

Statistical analysis

A Linear Mixed Model (LMM) was used to analyze the effect of rhizobacterium *Pf.* WCS417r inoculation on *M. brassicae* and *P. brassicae* performance followed by the least significant differences (LSD) post-hoc test. In experiments on P-grown *A. thaliana* plants, the model consisted of rhizobacteria treatment as a fixed factor and plant as a random factor. In experiments evaluating herbivore performance on *A. thaliana* Col-0 and mutant *dde2-2* plants the model included rhizobacteria treatment and plant line as fixed factors and plant as a random factor. In experiments comparing performance of *M. brassicae* in P-grown and 1P:1S-grown *A. thaliana*, the model contained rhizobacteria treatment and soil type as fixed factors and plant as a random factor. Due to the non-normality of the data, the results on gene transcript level were analyzed using Generalized Linear Models (GLM) with treatment (C, R, CM/CR, and RM/RP) as factor. The model was adjusted to a Poisson distribution with log link function, and the dispersion parameter was estimated to correct for over-dispersion. Data on rhizobacterial colonization density were analyzed using a GLM, with either soil type or plant line and time point as factors and number of bacteria (cfu.g^{-1} root fresh weight) as variable. All statistical analyses described above were performed in GenStat (14th Edition, VSN Int., UK). Correlation analysis was used to analyze data of *M. brassicae* larval weight and shoot dry weight in experiment 4 (IBM SPSS Statistics 19).

RESULTS

P. fluorescens WCS417r colonization of *A. thaliana* roots has a variable effect on performance of the generalist caterpillar *M. brassicae* and the specialist *P. brassicae*

In Experiment 1, rhizobacterial colonization of potting soil-grown *A. thaliana* Col-0 (N = 20) resulted in increased larval weight of *M. brassicae* at 6 dpi (F = 4.41, df = 1, P = 0.042) and 12 dpi (F = 7.2, df = 1, P = 0.011) (Fig. 1A). In contrast, in Experiment 2, conducted at a different time and using a different batch of the same commercial potting soil, rhizobacterial colonization negatively affected larval weight of *M. brassicae* at 6 dpi (F = 5.64, df = 1, P = 0.023) and 12 dpi (F = 12.68, df = 1, P = 0.001) on *A. thaliana* Col-0 plants (N = 20) (Fig. 2A). Colonization by rhizobacteria of the roots of *A. thaliana* Col-0 (N = 20) negatively affected larval weight of the specialist *P. brassicae* at 6 dpi (Fig. 1B; F = 15.9, df = 1, P < 0.001). However, at 10 dpi no effect of rhizobacterial colonization on larval weight was recorded (Fig. 1B; F = 0.04, df = 1, P = 0.847). In Experiment 2, rhizobacteria colonization had no effect on *P. brassicae* larval weight after 6 days of feeding (Fig. 2B; N = 20, F = 1.03, df = 1, P = 0.317). In Experiment 1, the density of rhizobacteria colonizing *A. thaliana* Col-0 plants (N = 5) was (6.18 ± 2.97) x 10^6 (mean ± SE) cfu.g^{-1} root fresh weight. In Experiment 2, the density of rhizobacteria colonizing *A. thaliana* Col-0 plants (N = 5) was (14.9 ± 6.6) x 10^7 (mean ± SE) cfu.g^{-1} root fresh weight.

P. fluorescens WCS417r-mediated ISR against *M. brassicae* is JA-mediated

In Experiment 2, larval weight of both herbivore species increased when feeding on *dde2-2* plants compared to Col-0 plants (Fig. 2; LSD-test, P < 0.05). Reduced larval weight of *M. brassicae* on rhizobacteria-treated *A. thaliana* Col-0 plants (N = 20) after 6 and 12 days of feeding was eliminated in the mutant *dde2-2* (Fig. 2A; LSD-test, P < 0.05). In contrast to the generalist caterpillar, the performance of the specialist *P. brassicae* was not affected by rhizobacteria in Col-0, and also not in *dde2-2* plants (Fig. 2B; N = 20). *Pf.* WCS417r colonized the rhizosphere of 6-week old *A. thaliana* Col-0 plants (N = 5) at a lower level than in *dde2-2* plants (N = 5), however in 7-week-old plants, *Pf.* WCS417r colonized both genotypes at a similar level (GLM, plant line : df = 1, P = 0.015, deviance ratio = 7.42; time point: df = 1, P < 0.001, deviance ratio = 52.95) (Fig. S3.).

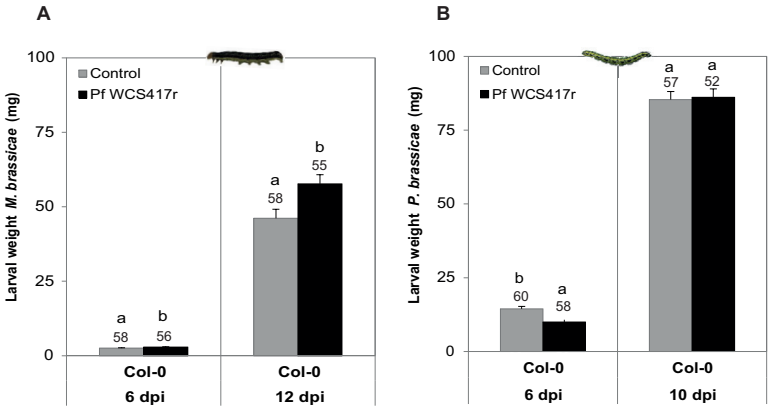

Figure 1. Performance of *Mamestra brassicae* (A) and *Pieris brassicae* (B) on control and rhizobacteria-treated plants of potting soil-grown *A. thaliana* Col-0. Panels A and B represent two experiments that were conducted at the same time using plants grown in soil from the same batch of potting soil. Three L1 larvae were placed on each plant (N = 20) and larval weight was measured at 6 and 12 dpi for *M. brassicae* performance and at 6 and 10 dpi for *P. brassicae* performance. Numbers above each bar represent the number of larvae surviving on the day of weight measurement. Data shown are means (± SE) of larval weight. Different letters above bars indicate significant differences between treatments (LMM, $P < 0.05$, LSD test).

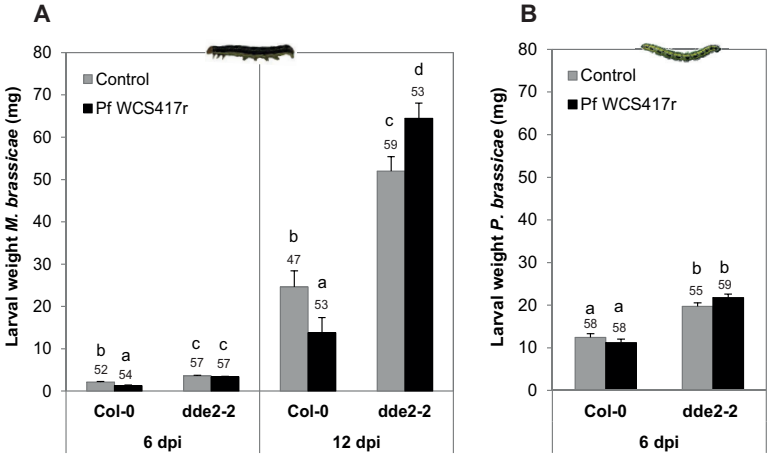

Figure 2. Performance of *M. brassicae* (A) and *P. brassicae* (B) on Col-0 and JA-biosynthesis mutant *dde2-2* plants. Panels A and B represent two experiments that were conducted at the same time using plants grown in the same batches of potting soil. Three L1 larvae were placed on each plant (N = 20) and larval weight was measured at 6 and 12 dpi for *M. brassicae* performance and at 6 dpi for *P. brassicae* performance. Numbers above each bar represent number of larvae suviving on the day of weight measurement. Data shown are means (± SE) of larval weight. Different letters above bars indicate significant differences between treatments (LMM, $P < 0.05$, LSD test).

P. fluorescens WCS417r colonization of *A. thaliana* induces enhanced expression of the defense-associated marker genes *LOX2* and *PDF1.2* upon caterpillar herbivory

Pf. WCS417r colonization (R) does not contribute to significant changes in both *LOX2* and *PDF1*.2 expression compared to that of control plants (C) (Fig. 3 A, B, C, D). In contrast, feeding damage of both herbivore species resulted in a significantly enhanced expression of *LOX2* and *PDF1.2* at 24 h and 72 h post infestation (GLM, $N = 5$, $P < 0.05$, LSD test; Fig. 3), with a higher expression at 24 h than at 72 h.

Interestingly, we observed that *Pf.* WCS417r colonization induced plants for an enhanced *LOX2* expression after 24 h of feeding by *M. brassicae* compared to control plants with *M. brassicae* feeding (GLM, $N = 5$, $P < 0.05$, LSD test; Fig. 3A). We also observed that *Pf.* WCS417r colonization induced plants for an enhanced expression of *PDF1.2* after 72 h of feeding by *P. brassicae* compared to 72 h of feeding on control plants (GLM, $N = 5$, $P < 0.05$, LSD pair-wise comparison; Fig. 3D). Additionally, transcript levels of *MYC2*, *VSP2*, *ERF1* and *ABA1* in *A. thaliana* upon feeding by either *M. brassicae* or *P. brassicae* were assessed and presented in supplementary materials (Fig. S1, S2). Feeding by either *M. brassicae* or *P. brassicae* resulted in a significant up-regulation of the defense-related genes *MYC2*, *VSP2* (at 24, 72 hpi) and *ABA1* (at 24 hpi) compared to control (uninfested) plants ($N = 5$, LSD-test, $P < 0.05$ for all five genes). No changes in *ERF1* transcript level were observed after 24 h and 72 h of feeding by either caterpillar species. *Pf.* WCS417r colonization does not induce enhanced expression of the four genes after 24 h or 72 h of feeding by *M. brassicae* or *P. brassicae* compared to feeding on control plants.

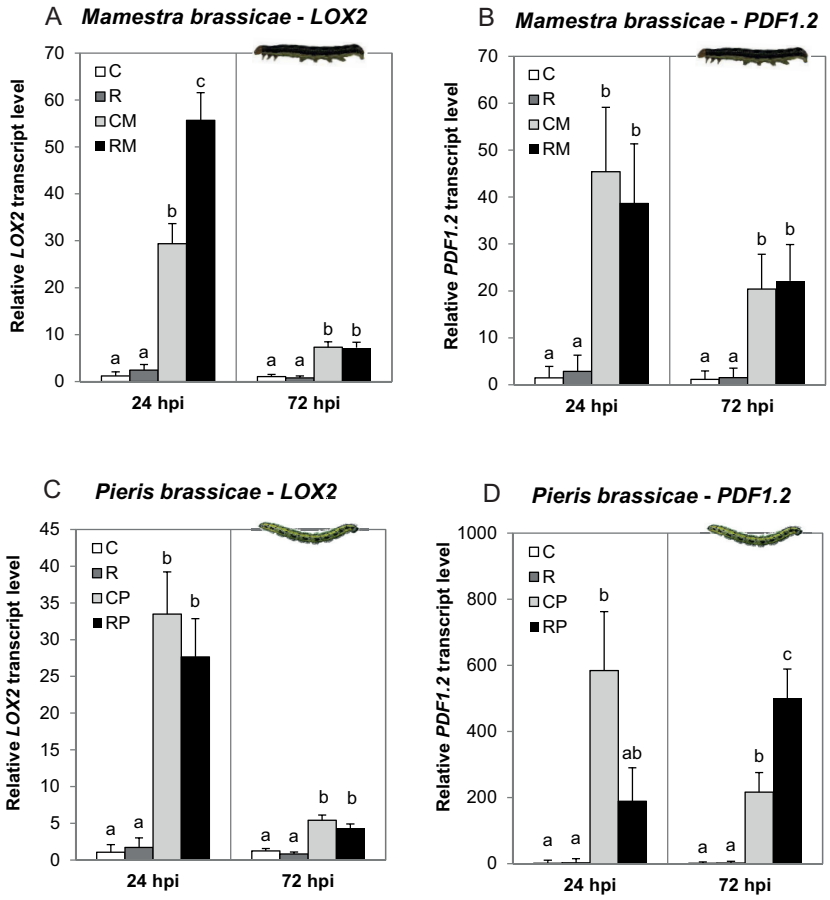

Figure 3. Relative transcript levels (mean ± SE) of defense-associated genes *LOX2* and *PDF1.2* in local leaves of *A. thaliana* Col-0 control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae*/ *P. brassicae* (CM/CP), or rhizobacteria-treated plants infested with *M. brassicae*/ *P. brassicae* (RM/RP) at 24 and 72 hpi. Plants were grown in potting soil. Values were normalized relative to the reference genes *EF* α and *FBOX*, and measured relative to the control plants ($N = 5$). Within each time point, different letters above bars indicate significant differences between treatments (GLM, $P < 0.05$, LSD test).

Soil composition contributes to the variation in the plant-mediated effect of *P. fluorescens* WCS417r colonization on performance of a generalist caterpillar

In a first trial, the effect of rhizobacteria colonization on caterpillar growth depended on the soil composition (LMM, $N = 25$**,** treatment x soil : $F = 7.5$, $df = 1$, $P = 0.007$) (Fig. 4A). In P-grown plants, rhizobacterial colonization of *A. thaliana* Col-0

increased larval weight of *M. brassicae* after 6 dpi. In contrast, in 1P:1S-grown plants, rhizobacterial colonization showed a trend toward lower larval weight. In a second trial (Fig. 4B) rhizobacterial colonization reduced larval weight of the generalist *M. brassicae* after 6 days of feeding independently of soil composition (LMM, $N = 25$, treatment: $F = 32.43$, $df = 1$, $P < 0.001$; treatment x soil $F = 0.71$, $df = 1$, $P = 0.402$). Correlation analysis of *M. brassicae* larval weight and shoot dry weight from both trials showed that only 4.2% of the variation in caterpillar growth can be explained by variation in plant biomass. In the first trial, the density of rhizobacteria colonizing *A. thaliana* Col-0 roots of P-grown plants was 1.77 (± 0.40) x10^7 (mean ± SE) cfu.g^{-1} root fresh weight, and on 1P:1S- grown plants this was 1.51 (± 0.37) x10^7 (mean ± SE) cfu.g^{-1} root fresh weight; these values are statistically similar (GLM, $N = 5$, soil type : $df = 1$, $P = 0.648$, deviance ratio = 0.23). In the second trial, the density of rhizobacteria colonizing *A. thaliana* Col-0 roots of P-grown plants was 2.12 (± 1.78) x10^7 (mean ± SE) cfu.g^{-1} root fresh weight, and on 1P:1S- grown plants was 4.63 (± 2.61) x10^7 (mean ± SE) cfu.g^{-1} root fresh weight; these values are statistically similar (GLM, $N = 3$, soil type : $df = 1$, $P = 0.469$, deviance ratio = 0.64).

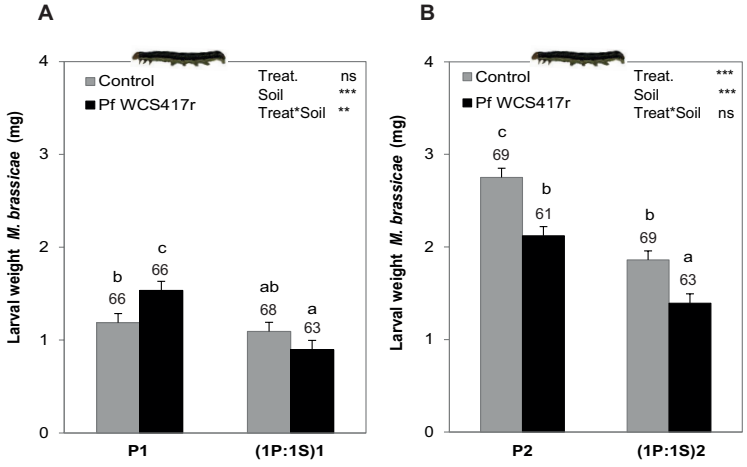

Figure 4. Performance of *Mamestra brassicae* on control and rhizobacteria-treated *A. thaliana* Col-0 plants grown in potting soil- (P) and a mixture of potting soil and sand (1P:1S). Panels A and B represent two experiments that were conducted at different times using plants grown in different batches of potting soil. Three L1 larvae were inoculated on each plant ($N = 25$) and larval weights were measured after 6 days of feeding. Numbers above each bar represent number of larvae surviving on the day of weight measurement. Data shown are means (± SE) of larval weight, and bar values having no letters in common differ significantly between treatments (LMM, $P < 0.05$, LSD test). The model consists of rhizobacterial treatment and soil type as fixed factors and number of plants as a random factor (*** $P < 0.001$; ** $P < 0.01$; ns, not significant).

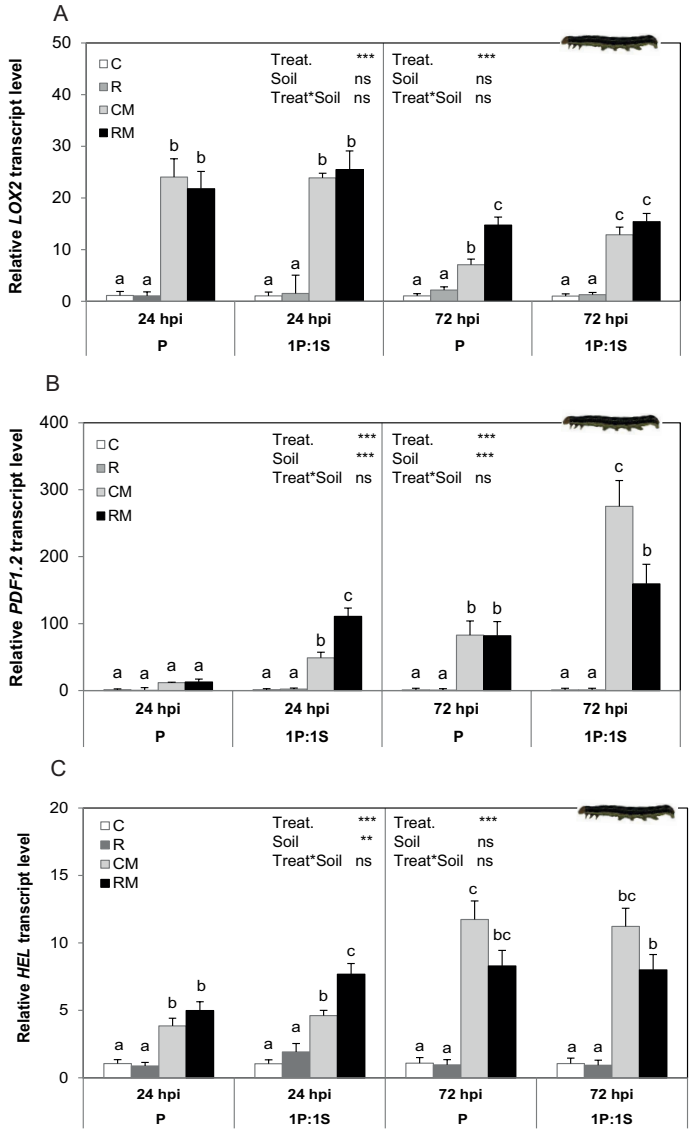

Figure 5. Relative transcript levels of *LOX2*, *PDF1.2* and *HEL* in local leaves of *A. thaliana* Col-0 control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), or rhizobacteria-treated plants infested with *M. brassicae* (RM) for 24 and 72 h. Plants were grown in potting soil (P) and a mixture of potting soil and sand (1P:1S). The experiment was performed at the same time and using similar batch of plants as in fig. 2B. Transcript levels (mean ± SE) of tested genes which were normalised relative to reference gene *EF1α* and *FBOX*, and measured relative to the control plants ($N = 5$). Within each time point, different letters above bars indicate significant differences between treatments (GLM, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, LSD test).

Soil composition had a significant effect on the rhizobacteria-triggered enhanced expression of the genes *PDF1.2* and *HEL* after 24 h of *M. brassicae* feeding, with a stronger induction on 1P:1S-grown plants than on P-grown plants (N = 5, LSD test; Fig. 5). *Pf.* WCS417r colonization enhanced expression of the *LOX2* gene after 72 h of *M. brassicae* feeding on P-grown plants but not the 1P:1S-grown plants (LSD test; Fig. 5A). Additionally, soil composition had a strong effect on the effect of herbivory on the expression of the *PDF1.2* gene at both time points as well as for *HEL* at 24h, with plants showing a higher induction when grown on 1P:1S soil than on P soil.

DISCUSSION

The effects of non-pathogenic microbes on induced systemic resistance against herbivores have received increasing interest. However, a main obstacle to advance in this field is the variation that is encountered when studying microbe-plant-insect interactions. Here, we faced the variable effects of rhizobacteria on the interactions between plants and insect herbivores. We have shown that soil composition plays a role in this variation. Rhizobacterial colonization of roots in potting soil-grown plants resulted in either positive or negative effects on the growth of the generalist *M. brassicae,* in two separate experiments. The reduced larval weight of *M. brassicae* indicates that *Pf.* WCS417r-mediated ISR was triggered in these plants. However, the effect was not consistent since under some conditions *Pf.* WCS417r colonization triggered induced systemic susceptibility (ISS). When evaluating the effect of soil composition on this variation, we showed that when the potting soil was diluted with sand in a 1:1 ratio, rhizobacterial treatment had a consistent negative effect on *M. brassicae* weight gain. In all cases the density of *Pf.* WCS417r that colonized *A. thaliana* roots was above the threshold required for initiation of ISR (Raaijmakers *et al.*, 1995), suggesting that the level of rhizobacterial colonization is not determining the observed variation.

Several soil characteristics may determine the observed variation, such as nutrient level, temperature or pH. Soil factors such as pH and temperature have been shown to affect the production of certain secondary metabolites by the soil-borne microbe *Pseudomonas chlororaphis* (Van Rij *et al.*, 2004), and these changes may affect microbe-plant interactions. Based on a meta-analysis of plant-mycorrhizae associations, the absence of nitrogen fertilization triggers more intense plant responses to mycorrhizal colonization (Hoeksema *et al.*, 2010). In fact, the main difference between the two soils evaluated here was their nutrient levels because sand is a substrate with a very low nutrient content, therefore nutrient level was probably reduced to 50% in the 1P:1S soil.

Together, this indicates that at relatively low nutrient levels, induction of rhizobacteria-mediated ISR is more consistent than at high nutrient level. Such consistency has also been observed in relatively low nutrient artificial media (N. Pangesti, M. Reichelt, J.E. van de Mortel, E. Kapsomenou, M. Dicke, J.J.A. van Loon, A. Pineda, unpublished results). In addition to soil composition, another element that may affect microbe-plant-insect interactions is volatile organic compounds (VOCs). It is known that several root-associated microbes produce VOCs that can trigger ISR (Ryu *et al.*, 2004). An interesting question that this study raises is how abiotic factors modulate microbe-plant signaling and what the consequences are for plant–insect interactions.

Interestingly, *Pf.* WCS417r colonization generally did not affect the performance of the specialist leaf-chewing insect *P. brassicae*, although a slightly reduced larval weight was observed at 6 days. The differential effect of rhizobacteria colonization on a generalist and specialist leaf chewer is in line with the characteristics of specialist insects that possess adaptations for detoxifying plant defensive compounds, in contrast to generalist insects that have a lower detoxification capacity for taxon-specific defense compounds (Schoonhoven *et al.*, 2005). Previous studies of mycorrhiza-plant systems have uncovered a general pattern in which the effect of interactions are positive or neutral for phloem feeders and specialist chewing herbivores, but negative for generalist chewing herbivores (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva *et al.*, 2009). Several studies of rhizobacteria-plant systems have shown similar patterns as in mycorrhiza-plant systems in which colonization of *P. fluorescens* WCS417r positively affected the generalist phloem feeders *Myzus persicae* (Pineda *et al.*, 2012), *Bemisia tabaci* (Shavit *et al.*, 2013); no effect was recorded on the performance of the specialist phloem feeder *Brevicoryne brassicae* (Pineda *et al.*, 2012), the specialist leaf chewing insect *Pieris rapae* (Van Oosten *et al.*, 2008), and a negative effect on the generalist leaf-chewing insect herbivore *Spodoptera exigua* (Van Oosten *et al.*, 2008). In fact in this study the effect of ISR on herbivore performance was maintained until pupation. Exceptions to the proposed general pattern on the effect of non-pathogenic microbes on plant-insect interactions are becoming more common in the literature. For instance, colonization of maize roots by the soil-borne endophytic bacterium *Enterobacter aerogenes* increased the growth rate of the generalist caterpillar *S. littoralis*, and this was speculated to be caused by changes in plant nutritional quality (D'Alessandro *et al.*, 2014). In conclusion, variation in the outcome of non-pathogenic microbe-plant-insect interactions has been mostly associated to biotic factors, and only recently the role of abiotic factors is starting to be demonstrated.

Despite the fact that rhizobacterial colonization of *A. thaliana* roots had a different impact on the performance of either *M. brassicae* or *P. brassicae,* both herbivores in general triggered similar plant responses at the transcriptional level. Herbivory by either *M. brassicae* or *P. brassicae* induced significant up-regulation of the defense-related genes *LOX2*, *PDF1.2, MYC2, VSP2* after 24 h and 72 h of feeding to a similar extent. When rhizobacteria were colonizing the plant, however, the plant responded faster and stronger to the attack of the generalist herbivore *M. brassicae* than to the specialist *P. brassicae*, as can be observed for *LOX2*. This phenomenon that allows plants to respond faster and stronger is known as priming and can also be induced following attack by pathogens or insects (Conrath *et al.*, 2006). Similar results were obtained in the first study reporting rhizobacterial priming of JA-regulated genes, where *PDF1.2* and *HEL* were more strongly induced by *S. exigua* than by *P. rapae* (Van Oosten *et al.*, 2008). In the present study, using the mutant *dde2-2* that is impaired in JA-biosynthesis, we further confirm that a functional JA-signaling pathway is required for rhizobacteria-mediated ISR against *M. brassicae*. The fact that *Pf.* WCS417r colonized the rhizosphere of *A. thaliana* Col-0 and *dde2-2* plants above the density required for induction of ISR (Raaijmakers *et al.*, 1995) indicates that rhizobacteria-mediated ISR is impaired in the *dde2-2* mutant due to altered characteristics of the mutant itself. The mutant *dde2-2* was also used to assess the role of JA signaling in the effect of rhizobacteria on herbivore-induced volatiles that are recognized by aphid parasitoids (Pineda *et al.*, 2013). Together, this indicates that a functional JA pathway is required for *Pf.* WCS417r-triggered changes in plant direct and indirect defense against insects.

The *Pf.* WCS417r-mediated enhanced expression of *PDF1.2* and *HEL* genes was stronger in plants grown in a mixture of sand and potting soil than in plants grown in potting soil, and soil composition significantly affected the expression of both genes. *PDF1.2* and *HEL* genes are controlled by a branch of the JA/ET pathway regulated by the ORA59 transcription factor; this branch is antagonistic to the MYC2-branch in the JA signalling pathway (Lorenzo and Solano, 2005). Intriguingly, we did not find rhizobacteria-mediated enhanced expression of the MYC2-branch marker gene *VSP2* after herbivory. Studies with mutants of the two JA branches are needed to investigate whether rhizobacteria can modify plant defense signaling to leaf-chewing insects by activating the ORA59 branch instead of the MYC2 branch. Moreover, since the 1P:1S soil is supposedly having only half the nutrient level, these results may imply that rhizobacteria-mediated priming of defense-related genes resulting in ISR is more pronounced in low-nutrient soils. Several recent studies show that soil nutrient status

can modulate signaling between plant and beneficial microbes from the early stage of the interaction. In mycorrhiza-plant interactions, combined limitation of both phosphate and nitrogen changes plant physiology and induces a transcriptomic profile indicating nutrient stress in *Medicago truncatula* roots favorable to mycorrhizal symbiosis (Bonneau *et al.*, 2013). Additionally, iron-limiting conditions also trigger high induction levels of the transcription factor MYB72 in *A. thaliana* plants (Palmer *et al.*, 2013), the same transcription factor that is crucial for the onset of *Pf.* WCS417r-mediated ISR (Van der Ent *et al.*, 2009). In soybean-rhizobia interactions, low nitrogen fertilizer treatment resulted in higher root nodulation, stronger accumulation of phytohormone *cis*-JA, compared to high nitrogen fertilizer treatment (Dean *et al.*, 2013). This may imply that at a low nutrient level, plants will be more dependent on root-associated microbes to obtain nutrients, and from this intense interactions microbes may trigger stronger ISR.

In studies of microbe-plant-insect interactions, variation in plant-mediated effects of root-associated microbes on insect herbivores is a main challenge. The lack of reproducibility in these multitrophic interactions may reflect our lack of understanding on factors affecting the study system (Heil, 2014). From the results presented in this study, we propose that soil nutrient level can potentially be one of the factors regulating the context-dependence in microbe-plant-insect interactions. The results show that induction of ISR is more consistent in a soil-sand mixture (i.e. with lower nutrient level among other differences), which is supported by transcriptional analyses demonstrating that the soil-sand mixture increased the magnitude of rhizobacteria-mediated expression of the ORA59-branch marker genes *PDF1.2* and *HEL* following herbivory. In nature, however, plants are exposed not only to insect herbivores, but also to natural enemies of the herbivores. It is therefore important to extend the study system and evaluate how rhizobacteria affect indirect plant defense against caterpillars to define the final positive or negative effects of these root-inhabiting microbes to plant fitness. Recent experimental findings indicate that the level of soil nutrients i.e. nitrogen, phosphate and iron are crucial in interactions between non-pathogenic rhizospheric microbes and plants (Bonneau *et al.*, 2013; Palmer *et al.*, 2013). Comprehensive studies of substrate ingredients and how these affect the level of rhizobacteria-mediated ISR against insects will help to predict conditions in which non-pathogenic microbes can benefit plant fitness most. Understanding what is driving the context-dependence in microbe-plant-insect interactions will be an important step towards application of these beneficial microbes in pest control.

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4

Rhizobacteria-induced systemic resistance against a leaf-chewing herbivore is associated with induction of aliphatic glucosinolates and the ORA59-branch of the jasmonic acid signaling pathway

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ABSTRACT

Beneficial microbes in the rhizosphere can promote plant defense and growth. Our previous study showed that a functional JA-signaling pathway is required for *P. fluorescens* WCS417r-induced systemic resistance (ISR) to the leaf-chewing insect *Mamestra brassicae*. Using *Arabidopsis thaliana* Col-0 and the same rhizobacterium, we here evaluate the role of the JA-regulated MYC2-branch and the JA/ET-regulated ORA59-branch in modulating rhizobacteria-mediated ISR to the leaf-chewing *M. brassicae* by combining gene transcriptional, chemical and herbivore performance assays. Whereas herbivory mainly induces the MYC2 branch, rhizobacterial colonization alone or in combination with infestation by the herbivore induced the expression of the defense-associated genes *ORA59* and *PDF1.2* at higher levels than activation by herbivore feeding alone, and the expression of both genes is suppressed in the knock-out mutant *ora59*. Interestingly, the colonization of plant roots by rhizobacteria alters the levels of the plant defense compounds glucosinolates (GLS) and camalexin, by enhancing the synthesis of constitutive and induced levels of camalexin and aliphatic GLS while suppressing the induced levels of indole GLS. The changes are associated with modulation of the JA-/ET-signaling pathways as shown by investigating mutants. This study shows a consistent effect of rhizobacteria-mediated ISR negatively affecting performance of *M. brassicae*, and functional JA and ET signaling pathways are required as observed in the knock-out mutants *dde2-2* and *ein2-1*. However, the transcription factors MYC2 and ORA59 are not required for the effect. We propose that colonization of plant roots by rhizobacteria modulates plant-insect interactions by prioritizing the ORA59-branch over the MYC2-branch, although the transcription factor ORA59 is not the only one responsible for the observed effects of rhizobacteria-mediated ISR against leaf-chewing insects.

Keywords: Plant growth-promoting rhizobacteria, caterpillar, signaling pathways, jasmonic acid, ethylene, glucosinolates, camalexin.

INTRODUCTION

Plants as primary producers in terrestrial ecosystems are exposed to various attackers, with insect herbivores among the most important ones. In their struggle to survive, plants evolved physical and chemical barriers as defenses against insect herbivores. Upon recognition of insect effectors, plants use hormones that regulate signaling pathways to reprogram their transcriptome and metabolome, thus strengthening their defense (Reymond *et al.*, 2004; De Vos *et al.*, 2005; Bodenhausen and Reymond, 2007). In brassicaceous plants, including *Arabidopsis thaliana*, glucosinolates (GLS) are the main defensive compounds that confer plant resistance against insect herbivores (Mewis *et al.*, 2006; Beekwilder *et al.*, 2008; Howe and Jander, 2008; Müller *et al.*, 2010). The two most abundant classes of GLS are aliphatic and indolic depending on whether the side chain is derived from the amino acid methionine or tryptophan respectively (Halkier and Gershenzon, 2006). It has been shown that feeding by specialist and generalist leaf-chewing insects triggers enhanced synthesis of aliphatic and indole GLS (Verhage *et al.*, 2011; Kos *et al.*, 2012), even though induction of indole GLS is stronger than aliphatic GLS (Kos *et al.*, 2012). More recent studies show that other compounds such as camalexin, a brassicaceous indolic phytoalexin, also contribute to plant resistance against insect herbivores (Kusnierczyk *et al.*, 2008; Schlaeppi *et al.*, 2008; Kettles *et al.*, 2013; Prince *et al.*, 2014). Most studies reported the sensitivity of phloem-feeding aphids to camalexin (Kusnierczyk *et al.*, 2008; Kettles *et al.*, 2013; Prince *et al.*, 2014). However, the effect on leaf-chewing herbivores is much less known. Unraveling how plant signaling pathways and crosstalk between the pathways regulate the synthesis of defensive compounds in the context of multitrophic interactions has only just begun.

The signaling pathway regulated by the plant hormone jasmonic acid (JA) is the core pathway regulating resistance to leaf-chewing herbivores (Farmer and Ryan, 1992; Kessler and Baldwin, 2002; Howe and Jander, 2008; Erb *et al.*, 2012), through formation of physical barriers such as trichomes and enhanced synthesis of defensive compounds such as GLS (Howe and Jander, 2008; Erb *et al.*, 2012). The JA-signaling pathway has two branches that cross-communicate with other hormonal pathways such as ethylene (ET) and abscisic acid (ABA) pathways through the use of common transcription factors (Lorenzo and Solano, 2005; Kazan and Manners, 2008). The transcription factor ORA59 is one of main integrators of the JA- and ET-signaling pathways (Lorenzo *et al.*, 2003; Pre *et al.*, 2008), whereas MYC2 is one of the main integrators of JA- and ABA-signaling pathways (Abe *et al.*, 2002), each of them activating different sets of JA-responsive genes (Lorenzo and Solano, 2005; Pieterse *et al.*, 2012; Kazan and

Manners, 2013). It has been shown that MYC2 suppresses the JA/ET-regulated branch (Dombrecht *et al.*, 2007). In *A. thaliana*, MYC2 regulates the biosynthesis of defensive compounds such as camalexin and GLS (Dombrecht *et al.*, 2007; Kazan and Manners, 2013; Schweizer *et al.*, 2013). In line with this, feeding by the leaf-chewing insects *Pieris rapae* and *Helicoverpa armigera* induced the MYC2-branch and increased the expression of the JA-responsive gene *Vegetative Storage Protein 2* (*VSP2*) (Dombrecht *et al.*, 2007; Verhage *et al.*, 2011). The MYC2-branch has also an important function in regulating plant interactions with beneficial microbes in response to pathogens (Pozo *et al.*, 2008). The next intriguing question is whether this MYC2 branch also regulates the plant-mediated interactions between beneficial microbes and insect herbivores.

Plants host a diversity of microbes, including beneficial microbes in the rhizosphere that can affect plant defense and growth. To establish interactions, beneficial microbes can modulate plant hormonal pathways regulating their colonization and triggering chemical modification in the plant (Verhagen *et al.*, 2004; Cartieaux *et al.*, 2008; Van de Mortel *et al.*, 2012; Weston *et al.*, 2012). For example, the JA signaling pathway regulates the colonization of *Medicago truncatula* by the mycorrhizal fungus *Glomus intraradices* and the nitrogen-fixing bacterium *Sinorhizobium meliloti* (Landgraf *et al.*, 2012). The plant hormone ET is also crucial in modulating the establishment of beneficial microbes, as was shown for the fungus *Piriformospora indica* in *A. thaliana* plants (Camehl *et al.*, 2010). Interestingly, several species of root-associated microbes from the genera *Pseudomonas*, *Bacillus*, and *Trichoderma* enhance plant immunity, through a mechanism called induced systemic resistance (ISR), known to inhibit growth and development of various insect herbivores and pathogens (Pineda *et al.*, 2010; Valenzuela-Soto *et al.*, 2010; Song *et al.*, 2013). Intact JA and ET hormonal signaling pathways are required to induce ISR by several root-associated microbes such as *P. fluorescens* WCS417r against pathogens (Pieterse *et al.*, 1998). It is unknown, however, how plants regulate chemical defense against insect herbivores upon colonization by root-associated beneficial microbes.

The present study investigates how colonization by the rhizobacterium *P. fluorescens* WCS417r affects the plant defense strategy against the leaf-chewing insect *Mamestra brassicae*. Previous studies found that this rhizobacterium triggers the enhanced expression of the JA-regulated gene *LOX2* and the JA/ET-regulated genes *PDF1.2* and *HEL* upon feeding by the generalist caterpillars *M. brassicae* and *Spodoptera exigua* (Van Oosten *et al.*, 2008; Pangesti *et al.*, 2015). A functional JA-signaling pathway is required for rhizobacteria-mediated ISR against the leaf-chewing insect *M. brassicae*

(Pangesti *et al.*, 2015). However, whether the JA-regulated MYC2-branch or the JA/ET-regulated ORA59-branch is modulating plant defense in rhizobacteria-mediated ISR against insects is unknown. To answer this question, analysis of gene transcription, plant chemistry and performance of the herbivore *M. brassicae* was performed *in vitro* in wild type *A. thaliana* Col-0 and mutants defective in the JA pathway, *dde2-2* and *myc2*, in the ET pathway, *ein2-1*, and in the JA/ET pathway, *ora59.* We hypothesize that rhizobacteria-treated plants 1) will trigger enhanced expression of the JA/ET-regulated genes *ORA59* and *PDF1.2* in wild type *A. thaliana* Col-0, whereas they will repress the JA-regulated genes *MYC2* and *VSP2* upon feeding by *M. brassicae,* 2) increase the synthesis of glucosinolates and camalexin upon feeding by *M. brassicae*, and 3) have stronger plant resistance to *M. brassicae* via the JA/ET-regulated branch ORA59 reflected by increased susceptibility to the herbivore in the *ora59* mutant.

MATERIALS & METHODS

Rhizobacterium *Pseudomonas fluorescens* WCS417r, growing conditions and quantification

The rifampicin-resistant, non-pathogenic rhizobacterium strain *P. fluorescens* WCS417r (abbreviated as *Pf.* WCS417r) was used in this study. Rhizobacteria were grown on King's B (KB) medium agar plates containing rifampicin (25 µg ml^{-1}) for 48 h at 28 °C (Pieterse *et al.*, 1996). Prior to inoculation on plant roots, a single colony of the strain was transferred to KB liquid medium amended with rifampicin as indicated above and was grown in an incubator shaker for 24 h at 200 rotations per minute (rpm) at 25 °C. The bacterial cells were collected, re-suspended in 10 mM $MgSO_4$, and washed three times with 10 mM $MgSO_4$. Afterwards, the bacterial cells were re-suspended in 10 mM $MgSO_4$ and adjusted to a cell density of 1 x 10^9 colony forming units (cfu) ml^{-1} (OD660 = 1.0).

Colonization of *A. thaliana* roots by *Pf.* WCS417r was quantified in wild type plants and mutants to confirm that the colonization met the required threshold for ISR of 10^5 cfu.g^{-1} root (Raaijmakers *et al.*, 1995). The rhizobacteria quantification was done following the method described in Pangesti *et al.* (2015), with slight modification. Roots were harvested, weighed and shaken vigorously for 1 min in 10 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (425-600 µm, Sigma-Aldrich). Proper dilutions were plated onto KB agar medium supplemented with 25 µg ml^{-1} rifampicin to select for rifampicin-resistant fluorescent *Pseudomonas* spp. (Pieterse *et al.*, 1998). The dilution plates were incubated for 48 h at 28 °C, after which the number of cfu per mg root fresh weight was determined.

Mamestra brassicae rearing

The generalist insect herbivore *M. brassicae* L. (Lepidoptera: Noctuidae; Cabbage moth) was reared on *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) in a climate chamber (22 ± 2 °C, 40 - 50 % RH, 16:8 h photo:scotophase). Newly-emerged larvae were used in the experiments.

Cultivation of *A. thaliana* Col-0 *in vitro*

Arabidopsis thaliana Col-0 plants were surface-sterilized and grown *in vitro* following a method described in Van de Mortel *et al.* (2012). In this study, *A. thaliana* Col-0 and mutants defective in the JA signaling pathway (*dde2-2*, *myc2)* and in the JA/ET signaling pathway (*ein2-1*, *ora59)* were used. Mutant *dde2-2* is defective in ALLENE OXIDE SYNTHASE, a key enzyme in the JA-biosynthesis pathway (Leon-Reyes *et al.*, 2010), mutant *myc2* is defective in transcription factor MYC2/JIN1 and is activated by the JA-signaling pathway (Hiruma *et al.*, 2011). Mutant *ein2-1* is defective in the nuclear protein ETHYLENE INSENSITIVE 2-1, a central component of the ET-signaling pathway (Alonso *et al.*, 1999; Wang *et al.*, 2007), mutant o*ra59* is defective in transcription factor ORA59 that is involved in the JA/ET-signaling pathways (Verhage *et al.*, 2011). A total of 12 seeds of the same line were sown on square plates (100 x 100 x 20 mm) (SARSTEDT, Nümbrecht, Germany) containing 50 ml of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), and seeds were incubated for 7 days in a growth chamber at 21 ± 2 °C, 60% relative humidity (RH), 16 h light : 8 h dark cycle, and 90 ± 1 µmol m^{-2} s^{-1} light intensity (SYLVANIA, GRO-LUX®, Germany). Seven-day-old plant seedlings were root tip-inoculated with 2 µl of *Pf.* WCS417r cell suspension (10^9 CFU ml^{-1}). For control treatment, plant seedlings were mock-inoculated with 2 µl of $MgSO_4$ solution. After root inoculation, plants were incubated for an additional 7 days in the same conditions as described above. Fourteen-day-old plants were used in the experiments.

Experiment 1.

Expression of marker genes of the ORA59- and MYC2-branch during rhizobacteria-mediated ISR

To test the hypothesis that rhizobacteria triggered enhanced expression of the JA/ET-regulated genes *ORA59* and *PDF1.2* in wild type *A. thaliana* Col-0, while repressing the JA-regulated genes *MYC2* and *VSP2* upon feeding by *M. brassicae*, we evaluated

gene expression of those four genes in *A. thaliana* Col-0 and mutants *myc2* (Hiruma *et al.*, 2011) and *ora59* (Verhage *et al.*, 2011). The four treatments of control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM) were arranged for each line. For each treatment, four to five biological replicates were used, each consisting of pooled leaves taken from four to five plates (each containing 11 to 12 seedlings) to ensure sufficient plant material for gene transcript analysis. Leaves were harvested at 24 h after insect infestation (hpi). Leaves of uninfested plants were treated and harvested similarly as those of infested plants. Leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C for further RNA extraction. Using the same batch of plants, performance of the caterpillars feeding on *Arabidopsis* wild type Col-0, and on the *myc2* and *ora59* mutants, and plant biomass were assessed as describe above.

Leaf samples were ground in liquid nitrogen and total RNA was extracted and purified following the protocol of RNeasy plant mini kit (Qiagen, Hilden, Germany). Measurement of RNA quality and procedure of cDNA synthesis followed methods described in Pangesti *et al.* (2015). Sequences of primers used in this study were *MYC2|JASMONATE INSENSITIVE1* (*MYC2*) (At1g32640) forward: 5'-ATCCAAGTTCTTATTCGGGTC-3' and reverse: 5'-CGTCTTTGTCTCTCTGCTTCG-3' (Pineda *et al.*, 2012); *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) (At5g24770) forward: 5'-TCAGTGACCGTTGGAAGTTGTG-3' and reverse: 5'-GTTCGAACCATTAGGCTTCAATATG-3' (Anderson *et al.*, 2004); *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* (*ORA59*) (At1g06160) forward 5'-TTCCCCGGAGAACTCTTCTT-3' and reverse 5'-GCCTGATCATAAGCGAGAGC-3' (Verhage *et al.*, 2011); *PLANT DEFENSIN 1.2* (*PDF1.2*) (At5g44420) forward 5'-CACCCTTATCTTCGCTGCTC-3' and reverse 5'-GTTGCATGATCCATGTTTGG-3' (Pineda *et al.*, 2012) and were quantified by qRT-PCR (CFX96™ Real-Time System, BIO-RAD, Hercules, CA, USA). Efficiency of each primer was determined before qRT-PCR analysis. Thermal cycling conditions consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 45 s. For each primer pair, controls without addition of template were performed to confirm that primer dimers were not interfering with detection of amplification. The transcript level for each tested gene was calculated relative to the reference genes *ELONGATION FACTOR 1α* (*EF1α*) (AT5G60390) with sequences of primers forward: 5'-TGAGCACGCTCTTCTTGCTTTCA-3' and reverse: 5'GGTGGTGGCATCCATCTTGTTACA-3' and *F-BOX*

FAMILY PROTEIN (*FBOX*) (AT5G15710) with sequences of primers forward: 5'-TTTCGGCTGAGAGGTTCGAGT-3' and reverse: 5'-GATTCCAAGACGTAAAGCAGATCAA-3' (Remans *et al.*, 2008) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Performance of *M. brassicae* and measurement of plant biomass

From the same batch of plants as for gene transcript analysis, additional plates were arranged to evaluate herbivore performance and plant biomass. Larvae that were feeding on *Arabidopsis* wild type Col-0, *myc2*, or *ora59* were weighed at 4 days post infestation (dpi), to the nearest 0.001 mg on a microbalance (CP2P, Sartorius AG, Germany). Afterwards, a pool of plant leaf material left in each replicate (squared plates) from control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* as well as control plants (C) and rhizobacteria-treated plants (R) were weighed to the nearest 0.1 mg (Mettler Toledo, Switzerland). Bioassays were performed in a growth chamber under similar conditions as described for plant cultivation.

Experiment 2.

Changes in glucosinolate and camalexin levels during rhizobacteria-ISR in the JA and ET-defective mutants *dde2-2* and *ein2-1*

To test the hypothesis that functional JA- and ET-signaling pathways are required for rhizobacteria to modify the synthesis of the plant defensive compounds GLS and camalexin, we evaluated the concentration of these compounds in *A. thaliana* Col-0, in the JA-biosynthesis defective mutant *dde2-2* (Von Malek *et al.*, 2002) and in the ET-signaling defective mutant *ein2-1* (Pre *et al.*, 2008). Using the same batch of plants, performance of the caterpillars feeding on *Arabidopsis* wild type Col-0, *dde2-2*, *ein2-1*, and plant biomass were measured as described above.

Glucosinolate and camalexin analysis

For glucosinolate (GLS) and camalexin analysis, four to five biological replicates were used, each consisting of pooled leaves taken from four to five plates (each containing 11 to 12 seedlings) to ensure sufficient material was collected for chemical analysis. Leaves were harvested at 4 dpi. Leaves of uninfested plants were treated and harvested at similar time points as those of infested plants. Leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C for further analysis. Leaf samples were ground to a

fine powder in liquid nitrogen and then lyophilized for 48 hours at -80 °C and pressure of < 10 mB.

Approximately 20 mg of lyophilized tissue were weighed for glucosinolates (GLS) analysis, and the exact weight of the tissue was recorded and used to calculate the GLS concentration. The GLS were extracted with 1 mL of 80 % methanol solution containing 0.05 mM intact 4-hydroxybenzylglucosinolate as internal standard and anlysed by HPLC-UV as described in Burow *et al.* (2006).

Camalexin was analysed in the flow-through samples resulting from the extraction procedure for GLS analysis. In GLS extraction, the raw extract is loaded onto DEAE Sephadex, the resulting flow-through when loading the extract was collected in a 96 deep-well plate and directly analysed by LC-MS/MS. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent, Germany). Formic acid (0.05 %) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-0.5 min, 5 % B; 0.5-1 min, 5-100 % B in A; 1-2 min 100 % B and 2.1-4. 5 min, 5 % B. The mobile phase flow rate was 0.8 ml/min. The column temperature was maintained at 25 °C. An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in positive ionization mode. The instrument parameters were optimized by infusion experiments. The ionspray voltage was maintained at 5500 V. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: m/z 201.09 →59.01 (collision energy (CE) 45 V; declustering potential (DP) 51 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of samples containing camalexin. Relative camalexin concentrations are expressed as peak area per mg weight.

Experiment 3.

Changes in glucosinolates and camalexin during rhizobacteria-mediated ISR in the transcription factor defective mutants *myc2* and *ora59*

To test the hypothesis that rhizobacteria modify the synthesis of plant defensive

compounds GLS and camalexin via the JA-/ET-regulated ORA59-branch, we evaluated the concentrations of the compounds in *A. thaliana* Col-0, ORA59-branch mutant *ora59* (Verhage *et al.*, 2011) and JA-regulated MYC2-branch mutant *myc2* (Hiruma *et al.*, 2011) plants as described for experiment 2. Using the same batch of plants, performance of the caterpillar feeding on *Arabidopsis* wild type Col-0, *ora59*, *myc2*, and plant biomass of all lines were measured as describe above. Analysis of GLS and camalexin content in plant shoots was performed as described for experiment 2.

Statistical analysis

Gene expression data were transformed (log(x+1)) and analyzed with one-way ANOVA to compare treatments within each line, whereas two-way ANOVA was used to compare treatments between lines. Glucosinolate data were analyzed with multivariate Projection to Latent Structures-Discriminant Analysis (PLS-DA) (SIMCA P+12.0, Umetrics AB, Umeå, Sweden). Analysis of individual and total aliphatic and indolic GLS were analyzed with one-way ANOVA to compare treatments within each line, whereas two-way ANOVA was analyzed to compare treatments between lines. Camalexin data were log-transformed and analyzed with one-way ANOVA to compare treatments within each line, whereas two-way ANOVA was analyzed to compare treatments between lines. *M. brassicae* performance data were analyzed with Linear Mixed Models (LMMs) within each line, with treatment as fixed factor and plate as random factor. Effect of rhizobacterial colonization on *M. brassicae* performance was also assessed between lines. Data of plant shoot and root biomass were analyzed with one-way ANOVA to compare treatments within each line, whereas two-way ANOVA was analyzed to compare treatments between lines.

RESULTS

Rhizobacterial colonization of *Arabidopsis thaliana* modifies plant signaling by prioritizing expression of genes in the ORA59-branch over those in the MYC2-branch

Transcript analyses of the JA-regulated gene *MYC2* show that this gene was affected by treatment and line, and there was an interaction between both factors (two-way ANOVA, treatment: $df = 3, 50$; $F = 5.75$; $P = 0.002$; line: $df = 2, 50$; $F = 28.50$; $P < 0.001$; treatment x line: $df = 6, 50$; $F = 3.09$; $P = 0.014$; Fig. 1A). In Col-0, feeding damage by *M. brassicae* on control plants (CM) and rhizobacteria-treated plants (RM) resulted in the up-regulation of *MYC2* in comparison to control plants (C). In the

mutants *myc2* and *ora59*, the expression of *MYC2* was lower in all treatments (R, CM, RM) in comparison to its expression in Col-0, and it was not induced by herbivory. Transcript analyses of the JA-responsive gene *VSP2* showed that its expression was affected by treatment but not line, and there was no interaction between treatment and line (two-way ANOVA, treatment: $df = 3, 50$; $F = 100.62$; $P < 0.001$; line: $df = 2, 50$; $F = 1.85$; $P = 0.170$; treatment x line: $df = 6, 50$; $F = 0.67$; $P = 0.677$; Fig. 1B). Similar to the expression of *MYC2*, in Col-0, feeding damage by *M. brassicae* on control plants (CM) and rhizobacteria-treated plants (RM) resulted in up-regulation of *VSP2* in comparison to control plants (C). In the mutants *myc2* and *ora59*, the expression of *VSP2* in all treatments (R, CM, RM) was comparable to its expression in Col-0, and was induced by herbivory. Overall, both *M. brassicae* feeding on control plants (CM) and on rhizobacteria-colonized plants (RM) induced the expression of the JA-regulated gene *VSP2* to a similar extent; however, the transcription factors MYC2 and ORA59 were not the only ones regulating the expression of both genes as shown in the *myc2* and *ora59* defective mutants.

Transcript analyses of the JA/ET-regulated gene *ORA59* showed that its expression was affected by treatment and line, but there was no interaction between both factors (two-way ANOVA: $df = 3, 50$; $F = 23.31$; $P < 0.001$; line: $df = 2, 50$; $F = 27.25$; $P < 0.001$; treatment x line: $df = 6, 50$; $F = 3.37$; $P = 0.009$; Fig. 1C). In *A. thaliana* Col-0 plants, rhizobacterial colonization with or without the herbivore (R and RM), and herbivory by *M. brassicae* (CM) resulted in up-regulation of *ORA59*. The expression of *ORA59* in R plants was higher than in CM and similar to RM. In the mutants *myc2* and *ora59*, the expression of the gene *ORA59* was lower in comparison to its expression in Col-0, but still was slightly but significantly induced by rhizobacteria colonization (R and RM treatments) although not by herbivory (CM treatment). Transcript analyses of the JA/ET-responsive gene *PDF1.2* showed that its expression was affected by treatment and line, and there was an interaction between both factors (two-way ANOVA: $df = 3, 50$; $F = 115.18$; $P < 0.001$; line: $df = 2, 50$; $F = 65.32$; $P < 0.001$; treatment x line: $df = 6, 50$; $F = 65.32$; $P < 0.001$; Fig. 1D). Similar to the expression pattern of *ORA59*, at 24 hpi in Col-0 plants, rhizobacterial colonization with or without herbivory (R and RM), and *M. brassicae* feeding (CM) resulted in up-regulation of *PDF1.2*, although the induction by rhizobacterial colonization was stronger than by herbivory. Interestingly, whereas in the mutant *myc2* the induction of *PDF1.2* in response to herbivory (in CM and RM treatments) was stronger than in Col-0, in the mutant *ora59* the induction of *PDF1.2* in response to herbivory (on CM and RM) was lower than in Col-0 plants. Taken together, rhizobacterial colonization with or without the herbivore (R and RM)

induced expression of JA/ET-regulated gene *PDF1.2* to higher levels than herbivore feeding (CM), and the expression of both genes was up-regulated to some extent by transcription factor *ORA59* as shown in the *ora59* defective mutant.

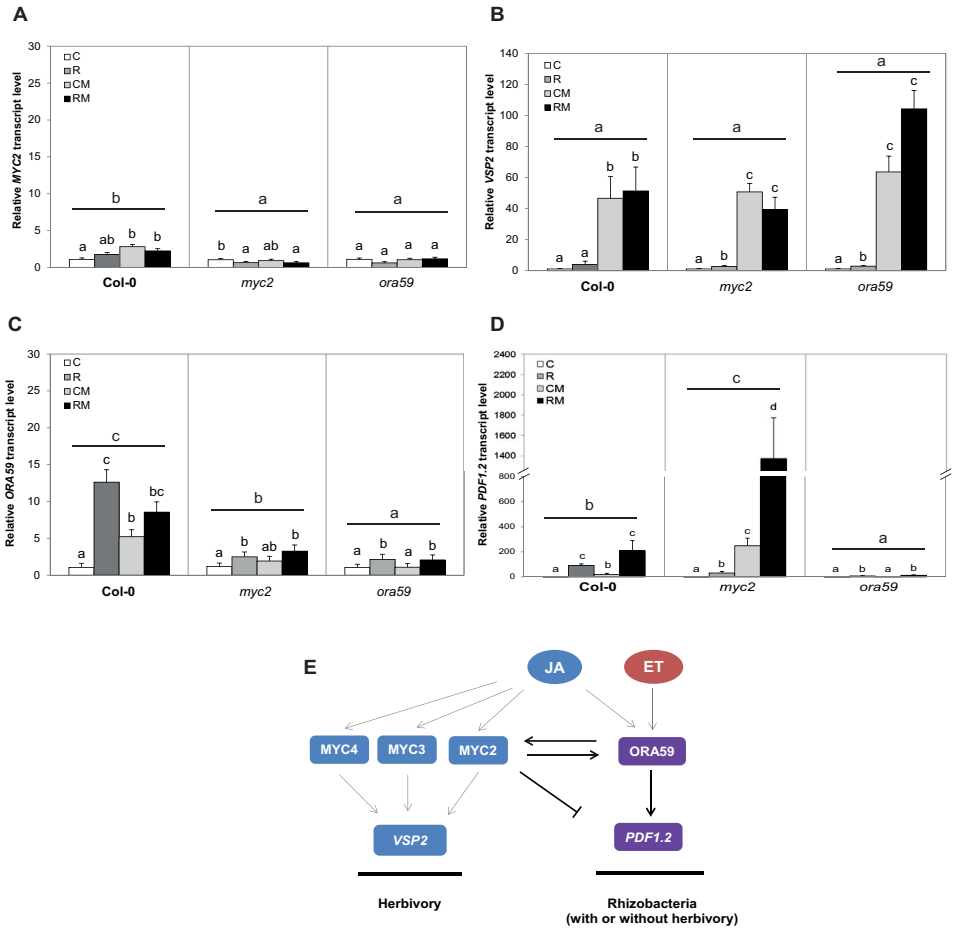

Figure 1. Relative transcript levels of *MYC2* (A), *VSP2* (B), *ORA59* (C) and *PDF1.2* (D). Expression of *MYC2* and *VSP2* genes are activated by the jasmonic acid (JA) pathway. Expression of *ORA59* and *PDF1.2* are activated by JA and ethylene (ET) pathways. Treatments are control plants (C), rhizobacteria-treated plants (R), control plants infested with two neonate larvae of *Mamestra brassicae* (CM), or rhizobacteria-treated plants infested with *M. brassicae* (RM) for 24 hpi. Transcript levels (mean ± SE) of tested genes were normalised relative to reference genes *EF1α* and *FBOX*, and measured relative to the control plants (N = 3-5 replicates, each from a pool of shoots collected from 3 to 5 plates). Different letters over the bars indicate significant differences within a line (one-way ANOVA, LSD post hoc test, $P < 0.05$), and letters over horizontal line indicate differences between lines (two-way ANOVA, LSD post hoc test, $P < 0.05$). (E) Working model of rhizobacterial induction of JA- and ET-regulated genes upon caterpillar feeding. Black arrows represent induction, truncated line represents suppression. Grey lines represent findings from previous studies (Verhage *et al.*, 2011; Schweizer *et al.*, 2013.).

Rhizobacterial colonization modifies the profile of total glucosinolates upon caterpillar feeding

In *A. thaliana* Col-0, a PLS-DA analysis of GLS in control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM) showed two significant principal components (PC) explaining 52.6. and 35.1% of the total variance, respectively (Fig. 2). The first PC (52.6%) separated the GLS based on the presence of caterpillars, whereas the second PC (35.1%) separated the GLS based on the presence or absence of rhizobacteria. In the shoot, a total of six aliphatic and four indole GLS were detected, and five GLS had a VIP value higher than 1. VIP values indicate the variable importance in the projection and those larger than 1 are the most influential for the model (Eriksson *et al.*, 2006). In decreasing order of importance, these compounds were 8MSOO (glucohirsutin), 7MSOH (glucoibarin), 1MOI3M (neoglucobrassicin), I3M (glucobrassicin), 4MTB (glucoerucin) (Table S3). The aliphatic GLS 4MTB was induced to the highest level in R plants, the indole GLS 1MOI3M and I3M were induced to the highest levels in CM, the aliphatic GLS 8MSOO and 7MSOH were induced the highest in RM plants. The analysis of GLS in *A. thaliana* Col-0 was repeated twice with similar results (Table S1, S2). In the mutants *dde2-2*, *ein2-1*, *myc2*, *ora59*, the GLS profile was separated based on the presence or absence of caterpillars or rhizobacteria in a similar way to Col-0 (Fig. S1).

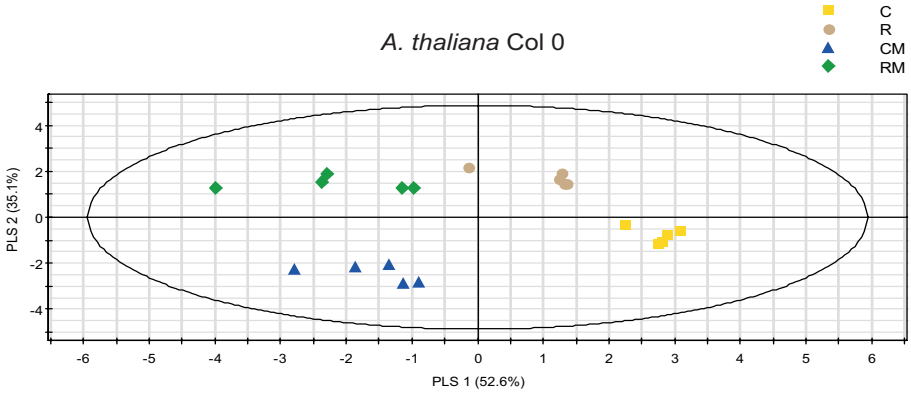

Figure 2. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparison of *Arabidopsis thaliana* Col-0 GLS profiles in shoots of control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), or rhizobacteria-treated plants infested with *M. brassicae* (RM). Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents a replicate (N = 5), each repliacte consisted of a pooled sample of *A. thaliana* shoots collected from 5 plates.

Rhizobacterial colonization modifies the profile of glucosinolates by enhancing the synthesis of constitutive and induced aliphatic GLS while suppressing the induced levels of indole GLS

A first experiment with *A. thaliana* Col-0, the JA-biosynthesis mutant *dde2-2* and the ET-insensitive mutant *ein2-1*, showed that total aliphatic GLS levels were affected by treatment, line, and the interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 18.59$; $P < 0.001$; line: $df = 2, 59$; $F = 98.94$; $P < 0.001$; treatment x line: $df = 6, 59$; $F = 8.83$; $P < 0.001$; Fig. 3A). Rhizobacterial colonization (R), *M. brassicae* feeding (CM), and the combination of both treatments (RM) induced synthesis of aliphatic GLS in Col-0, and such induction was reduced in *dde2-2* and *ein2-1.* A second experiment with *A. thaliana* Col-0, *myc2* and *ora59* plants showed that total aliphatic GLS were affected by treatment and line, but there was no interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 18.99$; $P < 0.001$; line: $df = 2, 59$; $F = 5.63$; $P = 0.001$; treatment x line: $df = 6, 59$; $F = 0.32$; $P = 0.92$; Fig. 3C). Rhizobacterial colonization (R), *M. brassicae* feeding (CM), and a combination of both treatments (RM) induced the synthesis of aliphatic GLS in Col-0, and such induction was slightly but significantly reduced in *myc2* and *ora59*. Taken together, the results show that *M. brassicae* feeding on rhizobacteria-colonized plants (RM) induced synthesis of aliphatic GLS to a higher level compared to *M. brassicae* feeding on control plants (CM), and both JA- and ET-signaling were required for the induced synthesis of aliphatic GLS as shown in the mutants *dde2-2*, *ein2-1*, *myc2*, *ora59*.

Total indole GLS in *A. thaliana* Col-0, and the mutants *dde2-2* and *ein2-1*, were affected by treatment, line, and the interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 166.68$; $P < 0.001$; line: $df = 2, 59$; $F = 224.12$; $P < 0.001$; treatment x line: $df = 6, 59$; $F = 45.18$; $P < 0.001$; Fig. 3B). Feeding by *M. brassicae* (CM) induced the synthesis of indole GLS, but in contrast to the levels of aliphatic glucosinolates, rhizobacterial colonization suppressed the synthesis of the indole GLS upon caterpillar feeding (RM). The synthesis of indole GLS was induced by JA-signaling, while it was suppressed by the ET-signaling pathway as shown in *dde2-2* where indole GLS was much suppressed, whereas in the *ein2-1* mutant it was increased. In a second experiment with *A. thaliana* Col-0, *myc2* and *ora59,* total indole GLS were affected by treatment, line, and the interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 60.13$; $P < 0.001$; line: $df = 2, 59$; $F = 6.58$; $P = 0.003$; treatment x line: $df = 6, 59$; $F = 2.81.$; $P = 0.02$; Fig. 3D). In Col-0, *M. brassicae* feeding (CM) induced the synthesis of indole GLS, whereas rhizobacterial colonization suppressed the synthesis of indole GLS upon caterpillar feeding (RM). The synthesis of indole

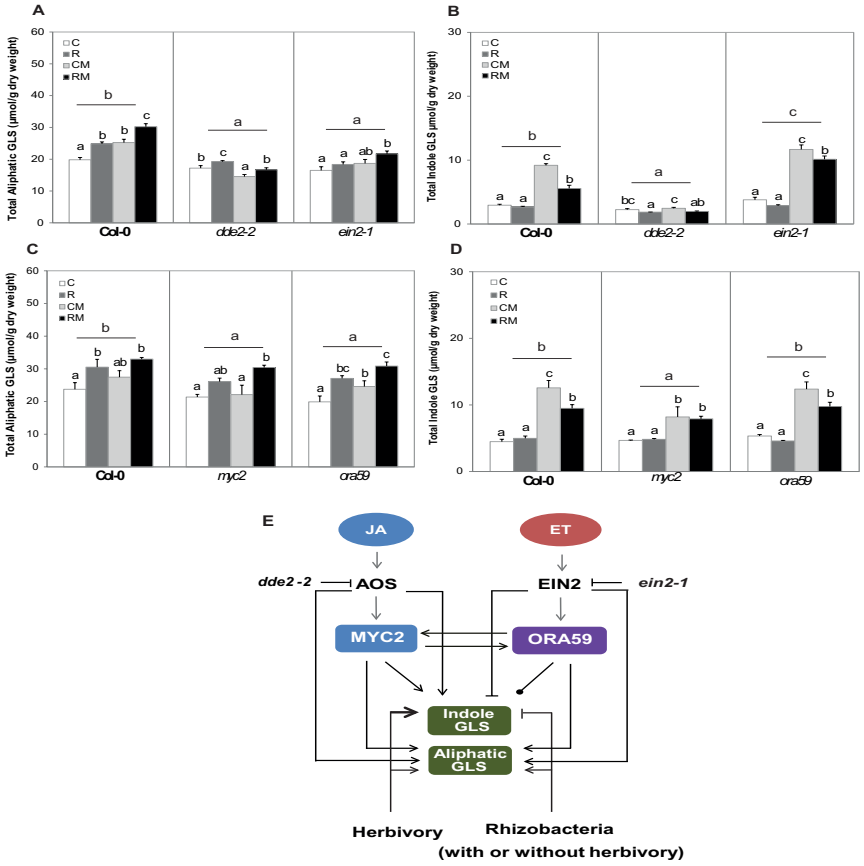

Figure 3. Total levels of aliphatic (A, C) and indole glucosinolates (B, D) in shoots of *A. thaliana* Col-0, and the mutants *dde2-2, ein2-1, myc2* and *ora59*. Treatments are control plants (C), rhizobacteria-treated plants (R), control plants infested with two neonate larvae of *M. brassicae* (CM), or rhizobacteria-treated plants infested with *M. brassicae* (RM) for 4 days ($N = 5$ replicates, each consisting of a pool of shoots collected from 5 plates). Different letters above the bars indicate significant differences within a line (one-way ANOVA, LSD post hoc test, $P < 0.05$), and letters above horizontal lines indicate differences between lines (two-way ANOVA, LSD post hoc test, $P < 0.05$). Panels A, B and C, D represent two different experiments. (E) Working model of rhizobacteria-triggered modification of GLS profile upon caterpillar feeding (RM) represented in black lines compared to control plant infested with caterpillar (CM) in *A. thaliana* represented in grey lines. Arrows represent induction, truncated lines represent suppression; dotted line indicates no effect. Grey arrows represent information from literature.

GLS was induced along the MYC2-branch, the ORA59-branch was not involved in induction or suppression of the synthesis of indole GLS because in *myc2* indole GLS were suppressed, while in *ora59* indole GLS were neither suppressed nor increased. Overall, *M. brassicae* feeding on control plants (CM) induced synthesis of indole GLS to

a higher level compared to feeding on rhizobacteria-colonized plants (RM), and induced synthesis of indole GLS was mediated by JA signaling as shown in mutants *dde2-2* and *myc2*. Whereas ET signaling suppressed the synthesis of indole GLS as shown in mutant *ein2-1*, the *ORA59* gene did not affect the synthesis of indole GLS.

Rhizobacterial colonization and herbivory-induced synthesis of camalexin

The concentration of camalexin in shoots of *A. thaliana* Col-0, the JA-biosynthesis mutant *dde2-2* and the ET-insensitive mutant *ein2-1*, was affected by treatment and line, and there was an interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 58.19.79$; $P < 0.001$; line: $df = 2, 59$; $F = 17.65$; $P < 0.001$; treatment x line: $df = 6, 59$; $F = 3.34$; $P = 0.008$; Fig. 4A). In Col-0, rhizobacterial colonization (R), *M. brassicae* feeding (CM), and the combination of both treatments (RM) induced camalexin, and the synthesis of camalexin was induced via JA-, but not via ET-signaling as shown in the *dde2-2* and *ein2-1* mutants.

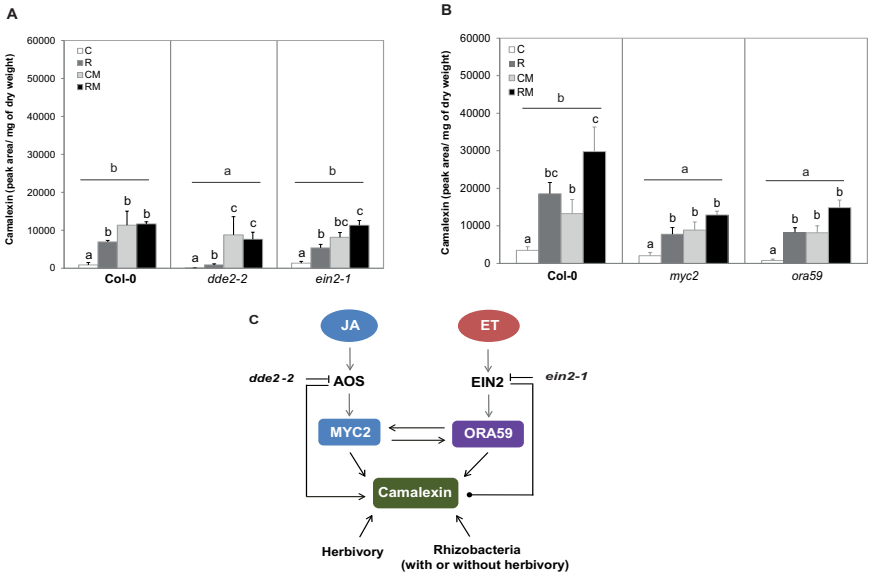

Figure 4. Concentration of camalexin (expressed in peak area units) in shoots of (A) *A. thaliana* Col-0 and the mutants *dde2-2* and *ein2-1*. (B) *A. thaliana* Col-0 and the mutants *myc2* and *ora59*. Treatments are: control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), or rhizobacteria-treated plants infested with two neonate larvae of *M. brassicae* (RM) for 4 days ($N = 5$ replicates, each composed of pooled shoots collected from 5 plates). Panels A and B represent two different experiments. Different letters over the bars indicate significant differences within a line (one-way ANOVA, LSD post hoc test, $P < 0.05$), and letters over horizontal line indicate differences between lines (two-way ANOVA, LSD post hoc test, $P < 0.05$). Arrows represent induction, dotted lines indicate that no effect was found.

The concentration of camalexin in shoots of *A. thaliana* Col-0, compared with the mutants *myc2* and *ora59* was affected by treatment and line, but there was no interaction between both factors (two-way ANOVA, treatment: $df = 3, 59$; $F = 37.88$; $P < 0.001$; line: $df = 9.15$; $F = 12.89$; $P < 0.001$; treatment x line: $df = 6, 59$; $F = 0.80$; $P = 0.575$; Fig. 4B). In Col-0, rhizobacterial colonization alone (R), herbivory (CM) or rhizobacterial colonization in combination with infestation by *M. brassicae* (RM) induced camalexin, and the synthesis of camalexin was induced by MYC2 and ORA59 as shown by the reduced levels in the mutants of both transcription factors.

Rhizobacteria-mediated induced systemic resistance against the generalist caterpillar *M. brassicae* requires functional JA and ET signaling pathways but is independent of MYC2 and ORA59

The performance of *M. brassicae* when feeding on *A. thaliana* Col-0, on the JA-biosynthesis mutant *dde2-2* and on the ET-insensitive mutant *ein2-1* in our *in vitro* system showed that the larval weight was affected by treatment and line, but there was no interaction between both factors (LMM, treatment: $df = 1, 103.5$; Wald stat. = 7.06; $P = 0.009$; line: $df = 2, 103.5$; Wald stat. = 20.16; $P < 0.001$; treatment x line: $df = 2, 103.6$; Wald stat. = 0.74; $P = 0.692$; Fig. 5A). In Col-0, rhizobacterial colonization of *A. thaliana* resulted in reduced larval weight of *M. brassicae* ($df = 1, 31.5$; Wald stat. = 4.94; $P = 0.034$). In contrast, when feeding on *dde2-2* and *ein2*-1, rhizobacterial colonization did not affect larval weight (*dde2*-2: $df = 1, 31.8$; Wald stat. = 1.87; $P = 0.181$; *ein2-1*: $df = 1, 31.3$; Wald stat. = 1.19; $P = 0.283$). Overall, the results show that rhizobacterial colonization resulted in reduced *M. brassicae* larval weight compared to control treatments, and functional JA- and ET-signaling were required for the rhizobacteria-mediated ISR against the herbivore as shown by the absence of an effect of rhizobacterial colonization in mutants *dde2-2* and *ein2-1*.

Performance of *M. brassicae* feeding on *A. thaliana* Col-0 and the mutants *myc2* and *ora59* showed that larval weight was affected by treatment and line, but there was no interaction between both factors (LMM, treatment: $df = 1, 484.6$; Wald stat. = 45.93; $P < 0.001$; line: $df = 1, 483.7$; Wald stat. = 7.12; $P = 0.029$; treatment x line: $df = 1, 484$; Wald stat. = 0.22; $P = 0.897$; Fig. 5B). Rhizobacterial colonization of *A. thaliana* Col-0 consistently resulted in reduced larval weight of *M. brassicae,* both in Col-0 ($df = 1, 77.4$; Wald stat. = 11.81; $P < 0.001$), and when feeding on *myc2* and o*ra59* (*myc2*: $df = 1, 84.5$; Wald stat. = 6.98; $P = 0.01$; *ora59*: $df = 1, 85.4$; Wald stat. = 7.77; $P = 0.007$). Data presented in fig. 5B are the combined results on *M. brassicae* performance from

experiments 1 and 3 (Fig. S2). In all experiments, the density of rhizobacteria colonizing the roots were above the required threshold for ISR induction (Table S4). Taken together, the rhizobacteria-mediated ISR resulted in reduced *M. brassicae* larval weight compared to control treatments, and the transcription factors MYC2 and ORA59 were not required for the effect as the effect remained the same in mutants *myc2* and *ora59*.

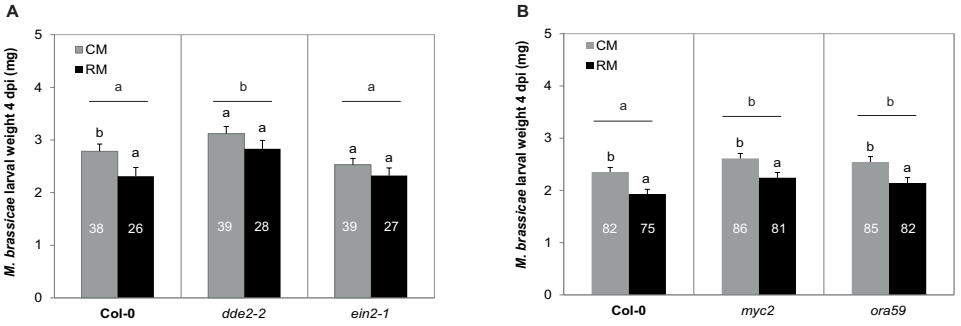

Figure 5. Performance of *M. brassicae* on control (CM) or rhizobacteria-treated plants (RM). Panels (A) (N = 14 – 23 plates) and (B) (N = 40 – 42 plates) represent two different experiments, and data of panel B are from two independent experiments (see Fig. S1 for extra information). Larval weight was measured at 4 dpi, after infesting each plate with two neonate larvae. Numbers in each bar represent number of larvae surviving on the day of weight measurement. Data shown are means (± SE) of larval weight. Different letters over the bars indicate significant differences within line, and letters over horizontal line indicate differences between lines (LMM, $P < 0.05$, LSD test).

Rhizobacterial colonization of the plant roots (R) had a strong effect on the shoot and root fresh weight in comparison to control plants (C). Under caterpillar attack, rhizobacteria-treated plants (RM) had stronger shoot and root growth in comparison to control plants infested with caterpillars (CM). In the mutants *dde2-2*, *ein2-1*, *myc2*, *ora59*, shoot and root biomass of plants in the four treatment groups showed a pattern similar to that of wild type plants (Fig. S3).

DISCUSSION

The present study shows a consistent effect of the rhizobacterium *P. fluorescens* WCS417r in triggering ISR that negatively affects the performance of the generalist caterpillar *M. brassicae*. The results show that while herbivory by this generalist herbivore prioritizes activation of the MYC2 branch of the JA-signaling pathway in the plant, rhizobacterial colonization causes a shift to a stronger activation of the ORA59-

branch (Figure 1). However, the transcription factor ORA59 is not the only responsible transcription factor for the observed effect of rhizobacterial colonization on caterpillar growth, based on the observation that the effect of rhizobacterial colonization on *M. brassicae* larval weight remained the same on the *ora59* mutant. Rhizobacterial colonization alone or combined with infestation by *M. brassicae* activated JA/ET-regulated *ORA59* transcription and increased the expression of the JA/ET-regulated marker gene *PDF1.2*. Using the same rhizobacterium-plant combination, previous studies found that rhizobacterial colonization of plant roots enhanced expression of *PDF1.2* only after herbivory (Van Oosten *et al.*, 2008; Pineda *et al.*, 2012; Pangesti *et al.*, 2015), a phenomenon known as "priming" of induced plant defense (Conrath, 2009). Interestingly, our results show that rhizobacterial colonization alone (R) induced expression of *ORA59* and *PDF1.2* to the same levels as in the combined treatment of rhizobacterial colonization and *M. brassicae* infestation (RM). A previous study in *A. thaliana* showed that attack by the necrotrophic pathogen *Botrytis cinerea* induced the expression of *ORA59* and *PDF1.2* in local and systemic leaves, and overexpression of *ORA59* increased plant resistance to the pathogenic fungus (Pre *et al.*, 2008). The gene *PDF1.2* encodes a plant defensin that is a basic peptide having antimicrobial activity against pathogens (Thomma *et al.*, 2002; Sels *et al.*, 2008). Here, we show that colonization by the beneficial rhizobacterium *P. fluorescens* WCS417r also induces the expression of *PDF1.2*, which suggests that the plant initially recognizes the rhizobacterium *P. fluorescens* WCS417r as a pathogenic organism, and therefore expresses defense-associated genes to limit its colonization. The results support a new interesting aspect of beneficial microbe-plant interactions, as it has been proposed that plants initially recognize the root-associated microbes as attacker, and therefore produce compounds that can limit the development of microbes (Pozo and Azcon-Aguilar, 2007; Zamioudis and Pieterse, 2012). Moreover, this requires mutual recognition and modulation of plant signaling by the beneficial microbes to establish mutualistic symbiosis with the plant (Pozo and Azcon-Aguilar, 2007; Pieterse *et al.*, 2014). To our knowledge, the role of defensin peptides such as PDF1.2 in plant defense to herbivorous insects is unknown, although it was recently proven that *P. rapae* oral secretion induces the expression of *PDF1.2* (Verhage *et al.*, 2011). Interestingly, recent studies highlight the importance of microbes in insect oral secretions or gut for the immune system and growth of herbivores as reviewed in Engel and Moran (2013), that can also influence plant-insect interactions. It requires further studies to investigate if the up-regulation of defensin peptides in plants colonized by rhizobacteria could negatively affect insect-associated microbes and suppress the insect's immune system, thus making the plant more resistant to insect herbivore.

The results show that rhizobacterial colonization enhanced the synthesis of constitutive aliphatic GLS as well as aliphatic GLS that are induced upon caterpillar herbivory, while suppressing the induced levels of indole GLS compared to non-colonized herbivore-infested plants. Moreover, rhizobacterial colonization or caterpillar feeding also induced synthesis of camalexin in the shoot. Using the same *in vitro* method, colonization of *A. thaliana* Col-0 roots by *P. fluorescens* strain SS101 induced accumulation of both aliphatic and indole GLS in the shoots and in the roots (Van de Mortel *et al.*, 2012), and upregulation of camalexin synthesis in local and systemic tissues of rhizobacterial colonized plants. Our present study and the study of Van de Mortel *et al.* (2012) indicate that different strains of a rhizobacterial species colonizing the roots could induce a unique GLS mixture in systemic tissues that may contribute to different strengths of ISR to herbivorous insects. The rhizobacterium *P. fluorescens* WCS417r used in this study is known to induce ISR against pathogens via the JA/ET signaling pathways (Pieterse *et al.*, 2002; Van der Ent *et al.*, 2009; Pieterse *et al.*, 2014), whereas ISR induced by *P. fluorescens* strain SS101 requires intact SA signaling (Van de Mortel *et al.*, 2012). Previous studies mostly indicated that camalexin is regulated via the SA pathway (Glazebrook, 2005; Glawischnig, 2007). The results of the present study show that the JA pathways and transcription factors MYC2 and ORA59 are also involved in the regulation of camalexin synthesis. In the case of GLS, differences in the pathways induced upon colonization by *P. fluorescens* WCS417r and *P. fluorescens* SS101 may drive the synthesis of different GLS. The experiments on mutants presented here show that activation of the JA signaling pathway induces the synthesis of aliphatic and indole GLS, whereas the ET pathway represses the synthesis of indole GLS. By modulating both the JA- and ET pathways, *P. fluorescens* WCS417r colonization alone and combined with caterpillar herbivory enhanced the synthesis of constitutive and induced aliphatic GLS and suppressed the synthesis of indole GLS. The sensitivity of *M. brassicae* to aliphatic GLS has been reported in a previous study (Beekwilder *et al.*, 2008), showing that in the *A. thaliana myb28myb29* double mutant lacking aliphatic GLS, the weight of *M. brassicae* increased 2.6 fold compared to the performance in wild type Col-0. Moreover, a negative correlation between the concentration of aliphatic GLS and performance of the generalist caterpillar *S. exigua* and the specialist *P. rapae* has been reported (Kos *et al.*, 2012). Taken together, we propose that induced accumulation of aliphatic GLS and suppression of indole GLS synthesis could be mechanisms underlying *P. fluorescens* WCS417r-mediated ISR against the leaf-chewing *M. brassicae* caterpillars. A study using mutants lacking aliphatic GLS may unravel the underlying mechanisms of rhizobacteria-plant-insect interactions.

Using a closed *in vitro* system, we here show that colonization by the rhizobacterium *Pf.* WCS417r had a consistent negative effect on the performance of the generalist caterpillar *M. brassicae* and this is associated with prioritization of the ORA59-branch over the MYC2-branch of the JA-signaling pathway, both constitutively and upon feeding by the generalist leaf-chewing *M. brassicae*. Using an open system in soil, we previously found that the effect of rhizobacterium *Pf.* WCS417r on plant direct defense against *M. brassicae* was variable, dependent on soil composition (Pangesti *et al.*, 2015). Moreover, it is known that in response to microbial attack and herbivore feeding, plants produce high levels of ET (De Vos *et al.*, 2005), although microbial attack induces higher levels of ET. In this study, experiments were conducted in a closed system, and it is possible that in the early stage of rhizobacterial colonization, plants emit ET and that accumulation of ET triggers prioritization of ORA59-branch even without caterpillar infestation. First, we propose that the prioritization of JA-/ET-targeted transcription factor ORA59, enhancing the synthesis of aliphatic GLS and suppressing the synthesis of indole GLS even without herbivore attack, and may strengthen rhizobacteria-mediated ISR against the caterpillar *M. brassicae*. However, mutation in the JA/ET-regulated *ora59*, which is downstream of *dde2-2* and *ein2-1*, does not have any effect on induction of rhizobacteria-mediated ISR against the caterpillars, suggesting that *ora59* alone does not explain rhizobacterial induced ISR against *M. brassicae*. Whether other JA-/ET-targeted transcription factors mediate the regulation of rhizobacteria-plant-insect interactions remains to be investigated. Secondly, we propose that simpler nutrient composition in the half-strength Murashige & Skoog (MS) media used in this study, compared to the nutrients available in soil, may result in more intense interactions between rhizobacterium and plant (Pangesti *et al.*, 2015), and could be one of factors that contributed to the consistent effect of rhizobacteria-mediated ISR against the generalist caterpillar *M. brassicae*. Moreover, in an open system other microbes can also colonize plant roots and compete with the rhizobacterium, and may therefore modify the rhizobacterium-plant interactions (Shavit *et al.*, 2013), which is not the case in the *in vitro* system used here.

In the present study we found evidence that colonization of plant roots by rhizobacteria alters plant-insect interactions at the level of gene transcription, plant chemistry and insect performance. Previous studies using the same rhizobacterium-plant combination recorded an up-regulation of the JA/ET-regulated genes *PDF1.2*, and *HEL* upon feeding by the caterpillars *Spodoptera exigua* (Van Oosten *et al.*, 2008), *M. brassicae* (Pangesti *et al.*, 2015), and the aphid *Myzus persicae* (Pineda *et al.*, 2012). This study

furthers our understanding of mechanisms of rhizobacteria-mediated ISR against leaf-chewing insects by showing not only that functional JA and ET pathways are required, but also that ISR against *M. brassicae* is induced along the ORA59 branch and not the MYC2 branch of the octadecanoid signal-transduction pathway. Furthermore, this study also provides new information on the induction of defensive compounds such as glucosinolates and camalexin by rhizobacterial colonization that can potentially explain the increased resistance to insect herbivores. Recent experimental evidence is uncovering the beneficial contribution of microorganisms to the functioning of humans, insects and plants by affecting growth, development, and immunity to diseases (Engel and Moran, 2013; Selosse *et al.*, 2014; Sugio *et al.*, 2014). In rhizobacteria-plant interactions, we found a higher expression of the *PDF1.2* gene that encodes a plant defensin peptide and secondary metabolites such as camalexin in rhizobacteria-colonized plants, that are known to have antimicrobial activity. This may be an initial response of plants to the recognition of the beneficial microbes. Whether these compounds have activity beyond antimicrobial effects, thus directly influencing insect physiology, or whether the compounds affect the insects indirectly by changing insect-associated microbes, thus modifying plant-insect interactions could be fruitful questions for future research to unveil the mechanisms underlying beneficial microbe-plant-insect interactions.

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5

Competitive interaction between beneficial *Pseudomonas fluorescens* strains in the rhizosphere does not affect the strength of plant growth promotion and induced systemic resistance against a leaf-chewing herbivore

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ABSTRACT

Non-pathogenic microbes residing in the rhizosphere may cooperate or compete, thereby affecting their collective benefit to the host plant. *Pseudomonas fluorescens* strains WCS417r and SS101 are well known for their ability to induce systemic resistance (ISR) in *Arabidopsis* via jasmonic acid (JA) or salicylic acid (SA) signaling pathways, respectively. Here, we evaluate how these strains interact in the rhizosphere of *Arabidopsis thaliana* Col-0 and how their co-cultivation affects plant growth promotion and plant defense to the leaf-chewing herbivore *Mamestra brassicae*. *P. fluorescens* WCS417r and SS101, applied individually to root tips or at two different positions along the roots, established similar population densities in the *Arabidopsis* rhizosphere. When co-cultivated at the same position in the rhizosphere, however, strain WCS417r established significantly higher population densities than SS101. Competitive interactions between the two strains were also observed in *in vitro* antagonism assays in the absence of plants, with WCS417 inhibiting growth of SS101. Both upon single inoculation and co-cultivation, the two strains induced the same level of ISR against the caterpillar *M. brassicae* and provided the same increase in plant biomass. At the plant transcriptional level, colonization by the two strains as single or mixed culture resulted in similar expression patterns of up-regulation of *MYC2*, down-regulation of *WRKY70* and no effect on *NPR1* expression, genes representative of JA signaling, SA signaling and the node of crosstalk between the two pathways, respectively. We hypothesize that both rhizobacterial strains use negative crosstalk between JA and SA pathways as mechanism to suppress plant immunity and establish colonization. This study shows that competitive interactions between rhizobacterial strains known to induce plant defense in systemic tissue via different signaling pathways, may interfere with synergistic effects on ISR and plant growth promotion.

Keywords: microbe-plant-insect interactions, microbial competition, *Mamestra brassicae*, rhizosphere, plant-growth promoting rhizobacteria.

INTRODUCTION

Symbiotic relationships between plants and beneficial microbes colonizing the roots are common in nature. To maintain the symbiosis, plants allocate significant amounts of plant photosynthetic carbon to the roots that can be used for microbial growth and reproduction (Denison *et al.*, 2003; Turner *et al.*, 2013). In return, the microbes often provide benefits to the host by enhancing plant growth and immunity (Jung *et al.*, 2012; Pieterse *et al.*, 2014). For instance, root-associated microbes such as plant growth-promoting rhizobacteria (PGPR) and mycorrhizae increase plant resistance through a mechanism called induced systemic resistance (ISR) which negatively affects growth and development of various insect herbivores (Bennett *et al.*, 2006; Koricheva *et al.*, 2009; Pineda *et al.*, 2010). Up to now, studies on beneficial microbe-plant-insect interactions have mainly focused on the use of single microbial species to achieve ISR (Pangesti *et al.*, 2013). Collaboration and competition between different groups of microbes i.e. bacteria-mycorrhizae and bacteria-rhizobia in the rhizosphere exists and may affect their collective benefit to host plants (Barea et al. 2005). For instance, microbial synergism has been observed in a network involving the bacterium *Paenibacillus* sp. that promotes the growth of the mycorrhizal fungus *Lactarius rufus,* colonizing *Pinus sylvestris* roots (Aspray *et al.*, 2006; Bonfante and Anca, 2009; Aspray *et al.*, 2013). Moreover, certain strains of root-associated *Pseudomonas* spp. increased nodule number on soybean roots colonized by the rhizobium *Bradyrhizobium japonicum* (Chebotar *et al.*, 2001). In contrast, intense competition between the two closely related mycorrhizal fungi *Rhizophagus* irregularis and *Glomus aggregatum* resulted in decreased total fungal abundance in experiments using *in vitro* root cultures (Engelmoer *et al.*, 2013). Furthermore, the direct interactions among microbes colonizing the same individual plant may alter the symbiotic relationships between the microbes and the plant (Denison *et al.*, 2003; Barea *et al.*, 2005), and may thereby also modify plant-insect interactions.

Interactions between a plant and its root-associated microbes are dynamic, and it has been suggested that the plant can actively shape the composition of its associated microbes (Doornbos *et al.*, 2012; Bulgarelli *et al.*, 2013; Turner *et al.*, 2013). The plant immune system is coordinated by interconnected hormonal signaling pathways, and hormones can also have a key role in determining plant microbiome structure (Turner *et al.*, 2013). For instance, activation of the jasmonic acid (JA) pathway in *Arabidopsis thaliana* altered the composition of carbon-rich root exudates leading to modification of rhizosphere bacterial communities (Carvalhais *et al.*, 2013). Furthermore, root-associated microbes can enhance plant immunity by modulating phytohormonal

signaling pathways via a mechanism known as induced systemic resistance (ISR) (Weller *et al.*, 2004; Van Wees *et al.*, 2008; Pieterse *et al.*, 2014). A growing body of evidence has shown that different strains of root-associated microbes can trigger ISR via JA/ET- or SA-dependent mechanisms, thus negatively affecting microbial pathogens and insect herbivores (Pieterse *et al.*, 1998; Van Wees *et al.*, 1999; Barriuso *et al.*, 2008; Van de Mortel *et al.*, 2012). SA- and JA-signaling pathways are well known to exhibit negative crosstalk, which may reduce plant resistance to leaf-chewing insects or plant pathogenic microbes (Leon-Reyes *et al.*, 2010; Soler *et al.*, 2012). Interestingly, a study on *A. thaliana* Col-0 showed that activation of both JA- and SA-dependent pathways induced by root colonization of *P. fluorescens* WCS417r and infiltration of the avirulent pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) carrying the *avrRpt2* gene into lower leaves respectively, resulted in an additive effect on the level of induced defense against the pathogen (Van Wees *et al.*, 2000). However, it is unknown whether simultaneous activation of JA- and SA-dependent resistance mechanisms induced by different species or strains of beneficial root-associated microbes will also result in enhanced plant resistance to insect herbivores and enhanced promotion of plant growth.

This study investigates how co-cultivation of two rhizobacterial strains, i.e. *P. fluorescens* WCS417r and *P. fluorescens* SS101, affects plant growth and plant direct defense to the leaf-chewing herbivore *Mamestra brassicae*. Previous studies showed that *P. fluorescens* WCS417r-mediated ISR in *Arabidopsis* operated via JA-regulated mechanisms and negatively affected the growth of the generalist leaf-chewing *Spodoptera exigua* and *M. brassicae* (Van Oosten *et al.*, 2008; Pangesti *et al.*, 2015). In contrast, *P. fluorescens* SS101 induced ISR in *Arabidopsis* via the salicylic acid (SA) pathway and negatively affected pathogenic *Pseudomonas syringae* pv *tomato* and the generalist leaf-chewing *S. exigua* (Van de Mortel *et al.*, 2012). Rhizobacteria in the genus *Pseudomonas* are among the most prolific root colonizers, which is an important trait for their beneficial effects on the plant (Lugtenberg *et al.*, 2001). However, how interactions between different species or strains of beneficial *Pseudomonas* affect root colonization and their effects on plant defense and plant growth has not been investigated in detail. Here we address the questions i) if co-cultivation of the two *P. fluorescens* strains affects colonization in the rhizosphere reciprocally, and ii) if co-cultivation affects plant-insect interactions and plant growth promotion. To answer these questions, the two *Pseudomonas* strains were either applied to roots of *A. thaliana* as single culture or as mixed culture, and their effects on root colonization, performance of the herbivore *M. brassicae* and plant shoot and root biomass were assessed. We also determined their effects on expression of the JA-

regulated gene *MYC2*, the SA-regulated gene *WRKY70* and the JA/SA-regulated gene *NPR1* in plant roots. We tested the hypotheses that co-cultivation of both strains will 1) affect the colonization level of each strain compared to single strain cultivation, 2) increase the strength of ISR against *M. brassicae* compared to single strain inoculation, and 3) increase plant growth compared to single strain inoculation.

MATERIALS & METHODS

Cultivation of *A. thaliana* Col-0 *in vitro*

Arabidopsis thaliana Col-0 plants were surface-sterilized for 3.5 h and grown *in vitro* following a method described by Van de Mortel *et al.* (2012). Twelve seeds were sown on square plates (100 x 100 x 20 mm) (SARSTEDT, Nümbrecht, Germany) containing 50 ml of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and incubated for 7 days in a growth chamber at 21 ± 2 °C and 60 ± 10 % RH under a 16 h light : 8 h dark cycle; 90 ± 1 µmol m^{-2} s^{-1} light intensity (SYLVANIA, GRO-LUX®, Germany). **In experiment 1**, seven-day-old plant seedlings were root tip-inoculated with 2 µl washed bacterial solution (OD_{660} = 1.0 ± 0.1) of either *Pf.* WCS417r or *Pf.* SS101. In the combination treatment, a mixture of two bacterial solutions in a 1:1 ratio (v/v) was applied. For control treatment, plant seedlings were mock-inoculated with 2 µl of $MgSO_4$ solution. **In experiment 2**, seven-day-old plant seedlings were inoculated with 2 µl washed bacterial solution (OD_{660} = 1.0 ± 0.1) of either *Pf.* WCS417r or *Pf.* SS101 at the root tips and at an area ca. 2 cm above the root tips. In two combination treatments, 2 µl bacterial solution of *Pf.* WCS417r was inoculated at the root tips, *Pf.* SS101 was inoculated at the area ca. 2 cm above the root tips and *vice versa*. After root inoculation, plants were incubated for an additional 7 days in the same condition as described above and used in an insect performance experiment.

Rhizobacteria growth conditions

The non-pathogenic rhizobacterial strains *P. fluorescens* WCS417r (abbreviated as *Pf.* WCS417r) and strain *P. fluorescens* SS101 (abbreviated as *Pf.* SS101) were used in this study. Both strains are resistant to rifampicin, resulting from spontaneous mutations, whereas strain *Pf.* SS101 is also resistant to kanamycin resulting from gene insertion at a neutral chromosomal site using the Tn7-based vector insertion method (Koch *et al.*, 2001). *Pf.* WCS417r was regularly cultured on King's B (KB) medium agar plates containing rifampicin (25 µg ml^{-1}) (Pieterse *et al.* (1996), whereas *Pf.* SS101

was maintained on King's B (KB) medium agar plates containing rifampicin (25 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) for 48 h at 25 °C. Prior to inoculation on plant roots, a single colony of each rhizobacterial strain was transferred to KB liquid medium amended with antibiotic(s) as indicated above and was grown in an incubator shaker for 24 h at 200 rpm at 25 °C. The bacterial cells were collected, re-suspended in 10 mM $MgSO_4$, and washed three times with 10 mM $MgSO_4$. Afterwards, the bacterial cells were re-suspended in 10 mM $MgSO_4$ and adjusted to a cell density of $1x10^9$ colony forming unit (cfu) ml^{-1} (OD660 = 1.0 ± 0.1).

Rearing of *Mamestra brassicae*

The generalist insect herbivore *M. brassicae* L. (Lepidoptera: Noctuidae; Cabbage moth) was reared on *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants in a climate chamber (22 ± 2 °C, 40 - 50 % RH, 16:8 h photo:scotophase). Newly-emerged larvae were used in the experiments.

Quantification of rhizobacteria *P. fluorescens* WCS417r and SS101 colonization

Roots were harvested 18 days after sowing or 11 days after rhizobacterial inoculation, weighed and shaken vigorously for 1 min in 10 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (425 - 600 μm, Sigma-Aldrich). To quantify *Pf.* WCS417r or *Pf.* SS101 from single strain treatments, proper dilutions of suspensions were plated onto KB agar medium supplemented with rifampicin (25 μg ml^{-1}) selective for the strain *Pf.* WCS417r or rifampicin (25 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) selective for *Pf.* SS101, respectively. To quantify both strains from the combination treatment, proper dilutions of suspension were plated onto KB agar medium supplemented with rifampicin (25 μg ml^{-1}), or with a combination of rifampicin (25 μg ml^{-1}) and kanamycin (100 μg ml^{-1}). Both strains grow in KB agar medium supplemented with rifampicin, whereas only strain *Pf.* SS101 survives in KB agar medium supplemented with rifampicin and kanamycin. Subtraction of the number of colonies counted on KB agar medium supplemented with rifampicin only with the number of colonies surviving in KB medium supplemented with rifampicin and kanamycin resulted in the number of *Pf.* WCS417r colonies. The dilution plates were incubated for 48 h at 25 °C, after which the number of cfu mg^{-1} root fresh weight was determined.

Antagonism assays between *Pf.* WCS417r and *Pf.* SS101 *in vitro*

To evaluate direct competition between the two *P. fluorescens* strains, an antagonism assay was carried out on KB agar plates amended with 25 μg mL^{-1} rifampicin. A rhizobacterium *Pf.* SS101 suspension of 50 μL (OD_{660}=1.0 ± 0.1) was homogenously spread over the surface of the plate, and afterwards a total of five 5 μL droplets of *Pf.* WCS417r were inoculated on the surface (see Figure 2). These plate assays were also executed *vice versa*, and observations were made of the bacterial growth.

Caterpillar performance bioassay *in vitro* and measurement of plant biomass

A. thaliana seedlings were used for insect performance bioassays in experiments 1 and 2 as described above. Two newly-hatched larvae of *M. brassicae* were transferred to each plate containing 12 seedlings using a fine paint brush, and sealed with plastic sealer (Darcopack, Ridderkerk, the Netherlands). One individual plate was counted as one biological replicate, and in total 20 - 25 replicates were used in each bioassay. The larvae were weighed at 4-days post infestation (dpi) to the nearest 0.001 mg on a microbalance (CP2P, Sartorius AG, Germany). Afterwards, a pool of plant leaf material left in each replicate (Petri dish) from control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM) as well as control plants (C) and rhizobacteria-treated plants (R) were weighed to the nearest 0.1 mg (Mettler Toledo, Switzerland). Bioassays were performed in a growth chamber under similar conditions as described for plant cultivation.

Gene transcript analysis in plant roots colonized by rhizobacteria

To evaluate whether the interaction between both strains can be explained by a plant-mediated mechanism, gene expression of *NPR1*, *MYC2*, *WRKY70* in the roots of rhizobacteria-treated plants was assessed. Four to five biological replicates were used for each treatment, each consisting of pooled roots taken from four to five plates (each containing 11 to 12 seedlings) to ensure sufficient material was harvested for gene transcript analysis. Roots were harvested at 11 day-post-inoculation (dpi). Root samples were immediately frozen in liquid nitrogen and stored at -80 °C for further RNA extraction. Root samples were ground in liquid nitrogen and total RNA was extracted and purified following the protocol of RNeasy plant mini kit (Qiagen, Hilden, Germany). Measurement of RNA quality and procedure of cDNA synthesis

followed methods described in Pangesti *et al.* (2015). Sequences of primers used in this study were *NON-EXPRESSOR OF PATHOGENESIS RELATED GENES1 NPR1* (At1g64280) forward: 5'-ACTTGACTCGGATGATATTGAG-3' and reverse: 5'- TAGTATCAATTGTGGCTCCTT-3'; *MYC2*/*JASMONATE INSENSITIVE1* (*MYC2*) (At1g32640) forward: 5'-ATCCAAGTTCTTATTCGGGTC-3' and reverse: 5'-CGTCTTTGTCTCTCTGCTTCG-3' (Pineda *et al.*, 2012); a transcription factor containing WRKY domain *WRKY70* (At3g56400) forward: 5'-CATGGATTCCGAAGATCACA-3' and reverse: 5'-CTGGCCACACCAATGACAA-3' (Besseau *et al.*, 2012). Efficiency of each primer was determined before qRT-PCR analysis. Thermal cycling conditions consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 45 s. For each primer pair, controls without addition of template were performed to confirm that primer dimers were not interfering with detection of amplification. The transcript level for each tested gene was calculated relative to the reference genes *ELONGATION FACTOR 1α* (*EF1α*) (AT5G60390) with sequences of primers forward: 5'-TGAGCACGCTCTTCTTGCTTTCA-3' and reverse: 5'GGTGGTGGCATCCATCTTGTTACA-3' and *F-BOX FAMILY PROTEIN* (*FBOX*) (AT5G15710) with sequences of primers forward: 5'- TTTCGGCTGAGAGGTTCGAGT-3' and reverse: 5'-GATTCCAAGACGTAAAGCAGATCAA-3' (Remans *et al.*, 2008) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistics

Data on root colonization by *Pf.* WCS417r and *Pf.* SS101 were log transformed and analyzed with one-way ANOVA. Data of *M. brassicae* performance were analyzed with Linear Mixed Models (LMMs) with treatment as fixed factor and plate as random factor. Data of plant shoot and root fresh weight were analyzed with two-way ANOVA comparing treatments with or without *M. brassicae* and with or without rhizobacteria as group factors. Data of gene expression were transformed (log(x+1)) and analyzed with one-way ANOVA to compare treatments. All data were analyzed using GenStat 16th edition, VSN International Ltd.

RESULTS

Rhizobacteria *P. fluorescens* strains WCS417r and SS101 compete in the rhizosphere of *A. thaliana* Col-0 and inoculation site affects the competition

Inoculation of *Pf.* WCS417r and *Pf.* SS101 as single cultures on *A. thaliana* Col-0 root tips resulted in similar levels of rhizobacterial colonization by each strain, whereas combined inoculation of both strains at the same site resulted in a significantly higher colonization level of *Pf.* WCS417r compared to *Pf.* SS101 (one-way ANOVA, $df = 3, 23$; $F = 20.18$; $P < 0.001$) (Exp. 1, Fig. 1A). The experiment was repeated twice yielding similar patterns (one-way ANOVA, $df = 3, 19$; $F = 376.71$; $P < 0.001$) (Supplementary material, Fig. S1).

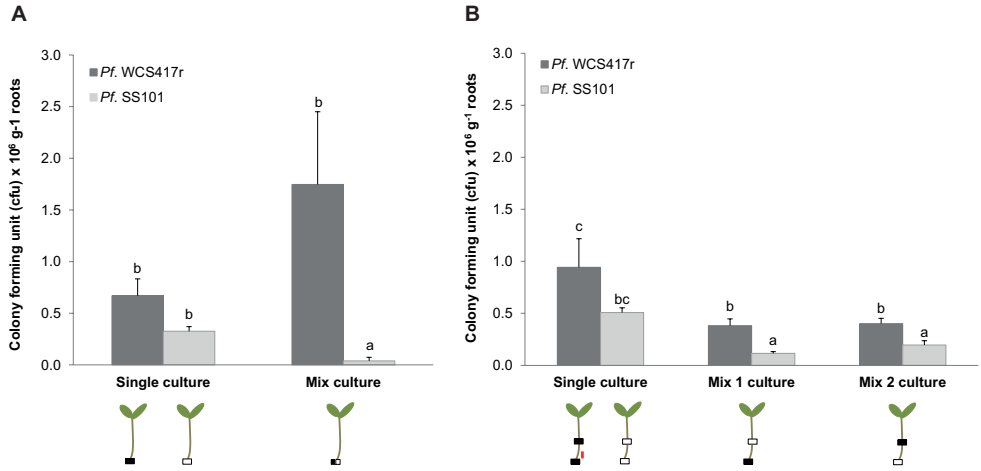

Figure 1. Abundance (mean ± SE) of *Pf.* WCS417r and *Pf.* SS101 in either single or combined inoculation of both strains expressed as colony forming units (cfu) mg^{-1} of root fresh weight. (A) Plant root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 suspension or a mixture of both strains at a ratio of 1 : 1 (v/v) ($N = 6$ replicates). (B) Inoculation was applied at root tips and at a zone ca. 2 cm above the root tips with 2 µl of 10 mM $MgSO_4$ for control, a suspension of *Pf.* WCS417r or *Pf.* SS101 or a mixture of both strains on each area ($N = 8$ replicates). Black rectangle, white rectangle, black-white rectangle in pictograms represent inoculations of *Pf.* WCS417r, *Pf.* SS101 and a mixture of both *Pf.* WCS417r and *Pf.* SS101 inoculations respectively. Vertical red line in 1B indicates 1 cm. Different letters above the bars indicate significant differences ($P < 0.05$, LSD test).

Inoculation of *Pf.* WCS417r and *Pf.* SS101 singly or as a mixture at different positions along the roots resulted in differences in rhizobacterial colonization by each strain (one-way ANOVA, $df = 5, 47$; $F = 11.28$; $P < 0.001$) (Exp. 2, Fig. 1B). Inoculation of *Pf.* WCS417r and *Pf.* SS101 as single cultures at different positions along the roots resulted in similar levels of rhizobacterial colonization by each strain, whereas combined inoculation of both strains at different positions along the roots resulted in significantly different colonization levels between the two strains.

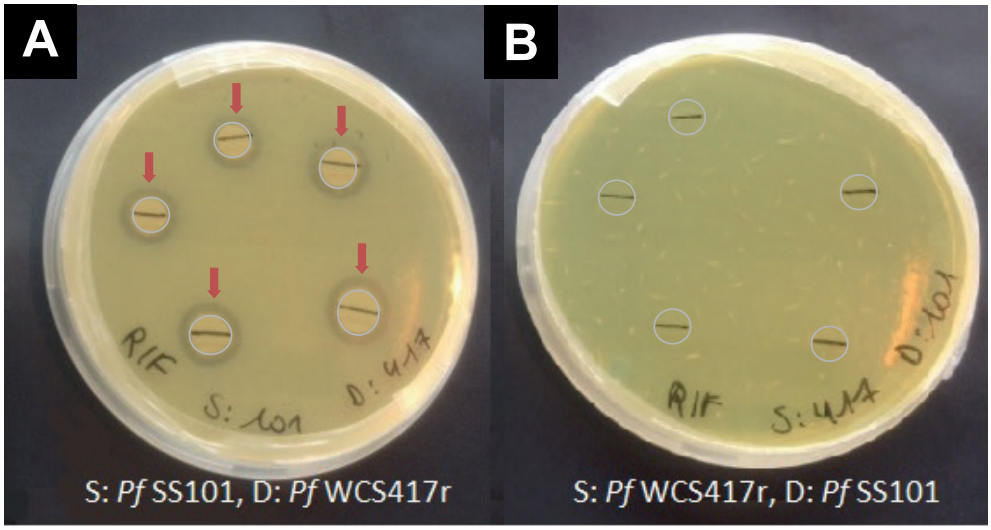

Figure 2. Antagonism assay between *Pf.* WCS417r and *Pf.* SS101 *in vitro*, pictures were taken 7 days post inoculation (dpi) for both strains. The two strains are grown on KB agar plates amended with 25 µg mL^{-1} rifampicin (RIF). **(A)** 50 µL of *Pf.* SS101 homogenously spread over the surface (S) and 5 dots (D) of *Pf.* WCS417r inoculated on the surface. **(B)** 50 µL of *Pf.* WCS417r homogenously spread over the surface (S) and 5 dots (D) of *Pf.* SS101 inoculated on the surface. Grey circles represent growing area of either *Pf.* WCS417r (A) or *Pf.* SS101 (B). Figure A shows clear inhibition zones (red arrows) indicating antibiosis exerted by *Pf.* WCS417r on *Pf.* SS101.

When *Pf.* WCS417r was spread over the plate surface and *Pf.* SS101 was inoculated as dots as shown in Fig. 2A, *Pf.* WCS417r created a clear zone of inhibition of growth of *Pf.* SS101 (Fig. 2A). Inversion of the surface and dot inoculation of the two strains did not result in a zone of inhibition. Therefore, whereas *Pf.* WCS417r has a negative effect on *Pf.* SS101 growth, *Pf.* SS101 has no effect on *Pf.* WCS417r growth.

Single or combined inoculation of rhizobacterial strains does not induce different strength of ISR against a leaf-chewing *M. brassicae*

In experiment 1, colonization of *A. thaliana* Col-0 roots by *Pf.* WCS417r, *Pf.* SS101, or the combination of both strains inoculated at the the same site resulted in reduced larval weight of *M. brassicae* at 4 dpi (df = 3, 88.7; Wald stat. = 26.83; P < 0.001) (Fig. 3A). Therefore, single or combined colonization of *Pf.* strains did not differ in its effect on larval weight. In experiment 2, colonization of *A. thaliana* Col-0 roots by *Pf.* WCS417r, *Pf.* SS101, or combination of both strains inoculated at different sites along the roots resulted in reduced larval weight of *M. brassicae* at 4 dpi (df = 4, 119.7; Wald stat. = 46.03; P < 0.001) (Fig. 3B). Therefore, single or combined colonization of *Pf.* strains did not differ in its effect on larval weight. Moreover, the positions of *Pf.* WCS417r and *Pf.* SS101 inoculation along plant roots did not affect the strength of plant resistance to the leaf-chewing herbivore.

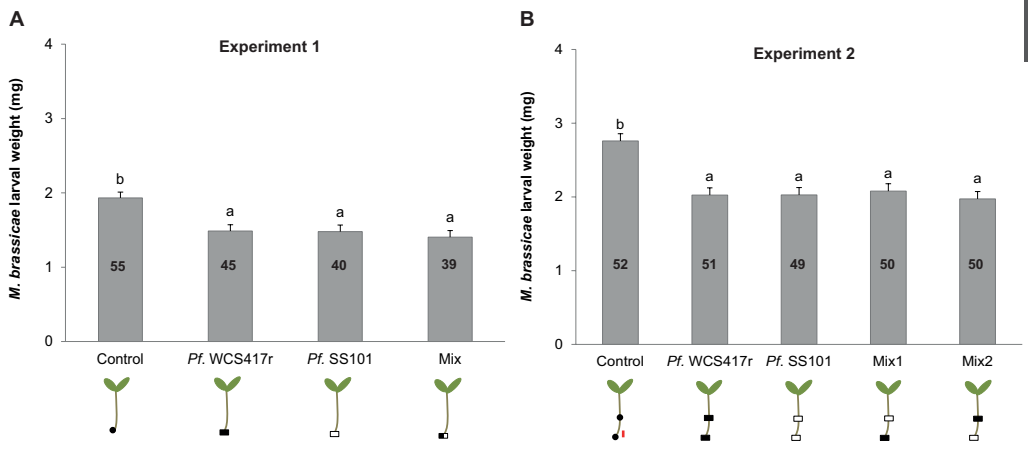

Figure 3. Larval weight of *M. brassicae* (means ± SE) on control and rhizobacteria-treated *A. thaliana* Col-0 plants at 4 days after infestation. **(A)** Plant root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, suspensions of *Pf.* WCS417r or *Pf.* SS101 or of a mixture of both strains at a ratio of 1 : 1 (v/v). **(B)** Plant root tips and a zone of approximately 2 cm above the root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 or mixture of both strains. Black circle, black rectangle, white rectangle and black-white rectangle in pictograms represent control, *Pf.* WCS417r and *Pf.* SS101 and a mixture of both *Pf.* WCS417r and *Pf.* SS101 inoculations respectively. Vertical red line in 3B indicates 1 cm. Numbers in each bar represent number of larvae surviving on the day of weight measurement. Different letters above bars indicate significant differences between treatments (LMM, P < 0.05, LSD test).

Single or combined inoculation of rhizobacterial strains do not differ in effects on plant biomass

Colonization of *A. thaliana* Col-0 roots by *Pf.* WCS417r, *Pf.* SS101, or the combination of both strains resulted in strongly increased shoot fresh biomass (two-way ANOVA, *Mamestra*: *df* =1; *F* = 7.89; *P* = 0.006; rhizobacteria: *df* = 3; *F* = 4.96; *P* = 0.003; Mam x rhizo: *df* = 3; *F* = 0.17; *P* = 0.919) (Exp. 1, Fig. 4A). In experiment 1, feeding by *M. brassicae* resulted in reduced shoot biomass, whereas colonization of plant roots by *Pf.* WCS417r resulted in statistically higher shoot biomass compared with control, whereas colonization of *Pf.* SS101 or combined colonization of both *Pf.* strains resulted in comparable shoot biomass to control. In experiment 2, feeding by *M. brassicae* also resulted in reduced shoot biomass, whereas inoculation of the two strains in different positions along the roots singly or in combination led to higher shoot biomass when compared to the control treatment (two-way ANOVA, *Mamestra*: *df* =1; *F* = 117.70; *P* < 0.001; rhizobacteria: *df* = 4; *F* = 41.56; *P* < 0.001; Mam x rhizo: *df* = 4; *F* = 2.52; *P* = 0.043) (Exp. 2, Fig. 4B). Root colonization by *Pf.* WCS417r singly resulted in higher shoot biomass compared to inoculation of *Pf.* SS101 singly. Whereas inoculation of *Pf.* SS101 resulted in similar shoot biomass compared to combined inoculation of both strains.

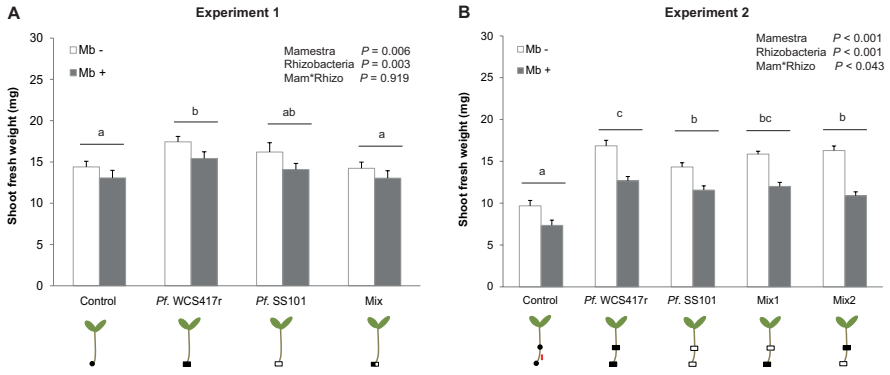

Figure 4. Shoot fresh weight (mean ± SE) of control and rhizobacteria-treated *A. thaliana* Col-0 plants without (Mb -) or with (Mb +) *M. brassicae* infestation. (A) Plant root tips were inoculated with 2 µl of 10 mM $MgSO_4$ for control, suspensions of *Pf.* WCS417r, *Pf.* SS101 or a mixture of both strains at a ratio of 1 : 1 (v/v). (B) Plant root tips and zone of app. 2 cm above root tips were inoculated by each 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 or mixture of both strains. Black dot, black rectangle, white rectangle and black-white rectangle in pictograms represent control, *Pf.* WCS417r and *Pf.* SS101 and a mixture of both *Pf.* WCS417r and *Pf.* SS101 inoculations respectively. Vertical red line in 4B indicates 1 cm. Comparisons are between treatments with or without *M. brassicae* and between control and rhizobacteria-treated treatments as group factors (two-way ANOVA, LSD post hoc test, *P* < 0.05).

Colonization of *A. thaliana* Col-0 plant roots by *Pf.* WCS417r, *Pf.* SS101, or the combination of both strains led to higher root biomass compared to the control treatment (two-way ANOVA, *Mamestra*: *df* =1; *F* = 1.66; *P* = 0.201; rhizobacteria: *df* = 3; *F* = 17.96; *P* < 0.001; Mam x rhizo: *df* = 3; *F* = 0.74; *P* = 0.531) (Exp. 1, Fig. 5A). In experiment 1, feeding by *M. brassicae* did not affect root biomass, whereas single inoculation by either *Pf.* WCS417r or *Pf.* SS101 compared to the combination of both strains at the root tip resulted in statistically similar root biomass. In experiment 2, all rhizobacteria treatments also led to higher root biomass compared to the control treatment (two-way ANOVA, *Mamestra*: *df* =1; *F* = 1.51; *P* = 0.222; rhizobacteria: *df* = 4; *F* = 86.12; *P* < 0.001; Mam x rhizo: *df* = 4; *F* = 0.21; *P* = 0.531) (Exp. 2, Fig. 5B). In experiment 2, feeding by *M. brassicae* did not affect root biomass, whereas colonization by *Pf.* WCS417r at two different positions along the root singly led to higher root biomass compared to the control and other rhizobacteria treatments. The combination of both strains resulted in higher root biomass compared to colonization by *Pf.* SS101 singly.

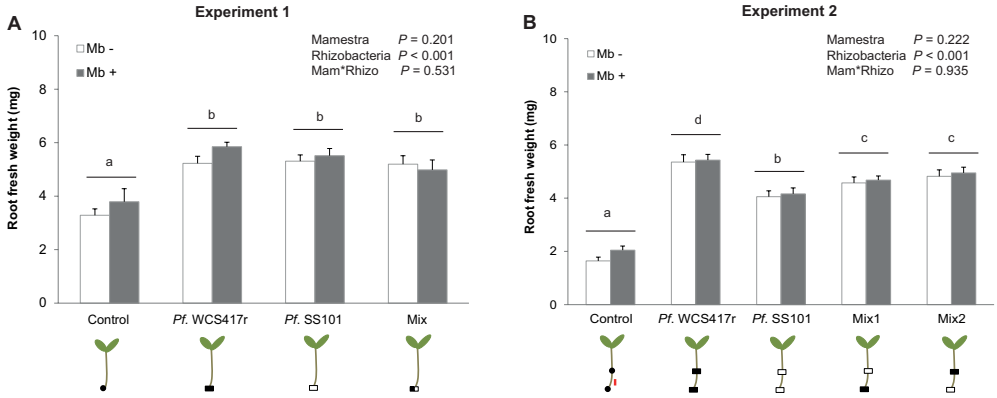

Figure 5. Root fresh weight (mean ± SE) of control and rhizobacteria-treated *A. thaliana* Col-0 plants without (Mb -) or with (Mb +) *M. brassicae* infestation. (A) Plant root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 or mixture of both strains at a ratio of 1 : 1 (v/v). (B) Plant root tips and zone of app. 2 cm above root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 or mixture of both strains. Black dot, black rectangle, white rectangle and black-white rectangle in pictograms represent control, *Pf.* WCS417r and *Pf.* SS101 and a mixture of both *Pf.* WCS417r and *Pf.* SS101 inoculations respectively. Vertical red line in 5B indicates 1 cm. Comparisons are between treatments with or without M. brassicae and between control and rhizobacteria-treated treatments as group factors (two-way ANOVA, LSD post hoc test, *P* < 0.05).

Rhizobacterial colonization by *Pf.* WCS417r and *Pf.* SS101 induces up-regulation of *MYC2* but down-regulation of *WRKY70* in *A. thaliana* roots

At day 11 after inoculation, *Pf.* WCS417r and *Pf.* SS101 colonization as single or mixed cultures resulted in up-regulation of *MYC2* gene transcription in plant roots in comparison to roots of control plants (Fig. 6). However, *Pf.* WCS417r colonization resulted in higher induction of *MYC2* transcription in comparison to *Pf.* SS101 in the roots (one-way ANOVA, $df = 3, 19$; $F = 154.22$; $P < 0.001$). Colonization by *Pf.* WCS417r and *Pf.* SS101 as single or mixed cultures resulted in down-regulation of *WRKY70* transcription in comparison to roots of control plants. All treatments resulted in a similar level of WRKY70 gene transcription (one-way ANOVA, $df = 3, 19$; $F = 7.31$; $P = 0.003$). In contrast, colonization by *Pf.* WCS417r and *Pf.* SS101 as single or mixed cultures resulted in similar levels of *NPR1* transcription in comparison to control plants (one-way ANOVA, $df = 3, 19$; $F = 0.49$; $P = 0.693$).

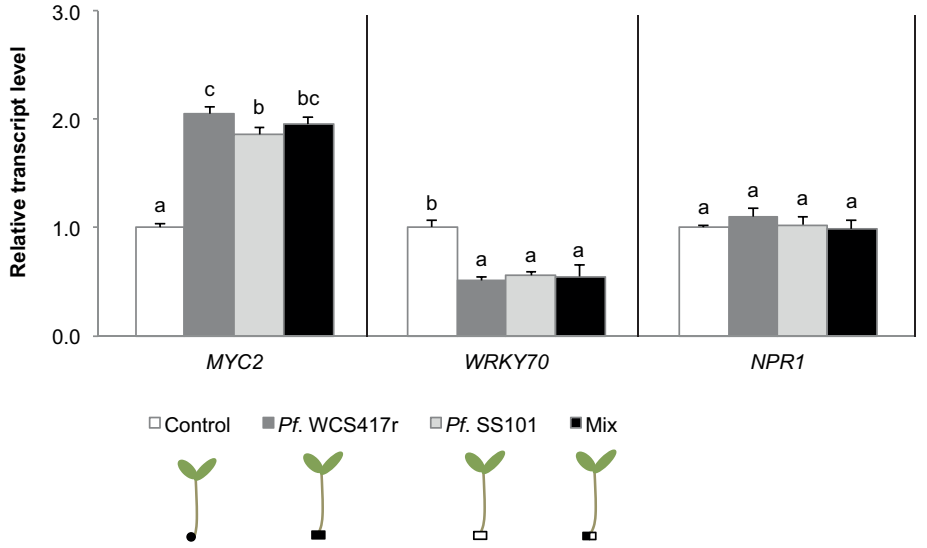

Figure 6. Relative transcript levels of *MYC2*, *WRKY70*, *NPR1* in the roots of control-, single *Pf.* WCS417r- or *Pf.* SS101-inoculated or mix-inoculated *A. thaliana* Col-0 plants at 18 days after sowing or 11 days after rhizobacterial inoculation. Transcript levels (mean ± SE) were normalized relative to reference genes *EF1α* and *FBOX*, and measured relative to the control plants ($N = 5$ replicates, each from a pool of roots collected from 3 to 5 plates). Black dot, black rectangle, white rectangle and black-white rectangle in pictograms represent control, *Pf.* WCS417r and *Pf.* SS101 and a mixture of both *Pf.* WCS417r and *Pf.* SS101 inoculations respectively. Different letters above bars indicate significant differences within each gene (one-way ANOVA, LSD post hoc test, $P < 0.05$).

DISCUSSION

This study shows that competition between two strains of root-associated *P. fluorescens* occurs in the rhizosphere with consequences for the abundance of each strain. Similarly, co-inoculation of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis* (previously known as *Glomus intraradices*) and *Glomus aggregatum,* were found to intensely compete, resulting in reduced abundance of each fungal species and also reduced overall fungal abundance (Engelmoer *et al.*, 2013). Interestingly, interactions between *Pf.* WCS417r and *Pf.* SS101 show a different pattern, when the two strains were co-cultivated at the same site, i.e. the root tip, the abundance of *Pf.* SS101 decreased, but the abundance of the *Pf.* WCS417r was similar to that after single inoculation. *In-vitro* antagonism assays in the absence of plants revealed that *Pf.* WCS417r was able to directly inhibit the growth of *Pf.* SS101. The root-associated bacterium *P. fluorescens* is well known to produce antibiotics that can limit the growth of other microorganisms (Raaijmakers *et al.*, 2010). However, to our knowledge it is unknown if *Pf.* WCS417r produces antibiotics that may be involved in their competitiveness in the rhizosphere. In interactions between plants and ectomycorrhizae, root tips are the site where nutrient exchange occurs and, therefore, competition among the fungi to colonize the root tips is high (Hoeksema and Kummel, 2003). Interestingly, the present study also shows that the site of inoculation influences the interactions between the two strains of root-associated *P. fluorescens*. Co-cultivation of both strains at the root tips resulted in a low level of *Pf.* SS101 colonization of 3.93×10^4 cfu g^{-1} root fresh weight, which is below the threshold (1×10^5 cfu g^{-1} root fresh weight) known to be required for induction of ISR in the plant against pathogen *Fusarium* in radish plants (Raaijmakers *et al.*, 1995a). Further study is needed to verify if the same threshold of rhizobacterial colonization also applies to induction of ISR against insect herbivores. Spatial separation of *Pf.* WCS417r and *Pf.* SS101 in an early stage of colonization allows each strain to sufficiently colonize the roots at a level above the known threshold for ISR induction. The results also show that *Pf.* WCS417r grew better than *Pf.* SS101 at different sites along the roots, which may suggest that *Pf.* WCS417r can use more diverse types of nutrients along the roots.

The results reveal that co-cultivation of *Pf.* WCS417r and *Pf.* SS101 does not result in a different strength of ISR induction against the herbivore *M. brassicae*, compared to single inoculation by each strain separately. Previous studies have shown that colonization by either *Pf.* WCS417r or *Pf.* SS101 singly induced plant resistance via JA- and SA-signaling pathways respectively, negatively affecting the growth of the generalist caterpillars *M. brassicae* and *S. exigua* (Van Oosten *et al.*, 2008; Van de Mortel *et al.*,

2012; Pangesti *et al.*, 2015). This study also shows that *Pf.* SS101 colonization increased plant resistance to *M. brassicae*, which is in line with a previous study showing a negative effect on another generalist caterpillar, i.e. *S. exigua* (Van de Mortel *et al.*, 2012). When both *Pf.* WCS417r and *Pf.* SS101 are inoculated at the same root site, only *Pf.* WCS417r colonization reaches the known threshold for ISR induction. Thus, it is possible that the effect on larval growth of *M. brassicae* upon combined inoculation is solely due to *Pf.* WCS417r-mediated ISR. Here, the results also show that in *A. thaliana* Col-0, single or co-inoculations of *P. fluorescens* strains WCS417r and SS101 at different positions along the roots, had a similar effect on induced plant resistance to the leaf-chewing *M. brassicae* as inoculation by either strain singly. It has been proposed that application of root-associated microbes that induce ISR via different signaling pathways, such as the JA and SA pathways, may increase the level of plant resistance by inducing biosynthesis of a higher diversity of secondary metabolites (Alizadeh *et al.*, 2013; Pangesti *et al.*, 2013). Using a different biological system, previous studies found that activation of both JA- and SA-signaling pathways can have different effects on the level of induced defense to plant attackers showing stronger (Van Wees *et al.*, 2000) or weaker (Leon-Reyes *et al.*, 2010; Soler *et al.*, 2012) resistance. It is known that crosstalk of the JA- and SA-pathways can yield synergistic or antagonistic effects on induced plant defense depending on the concentration of the hormones applied exogenously or the feeding mode (chewing or piercing-sucking) of the insect herbivores (Mur *et al.*, 2006; Kroes *et al.*, 2014; Wei *et al.*, 2014). It remains to be investigated if in the case of rhizobacteria-induced ISR via JA- and SA-signaling pathways, concentration-dependent mechanisms that may be induced by different densities of the rhizobacteria also play a role in regulating the induced plant defense against insect herbivores. Furthermore, *Pf.* WCS417r and *Pf.* SS101 colonization of plant roots are known to modify plant defense compounds, *e.g.* glucosinolates, in the shoot (Van de Mortel *et al.*, 2012; N. Pangesti, M. Reichelt, J.E. van de Mortel, E. Kapsomenou, J.J. A. van Loon, M. Dicke, A. Pineda, unpublished data). It is possible that combined inoculation of *Pf.* WCS417r and *Pf.* SS101 does not significantly modify the profile of glucosinolates or other defensive compounds that could result in a stronger or weaker resistance to the generalist leaf-chewing *M. brassicae*. It will be interesting to test the effect of combined inoculation of the two rhizobacterial strains on the hemibiotrophic pathogen *Pseudomonas syringae* DC3000, as it has been shown that this pathogen is sensitive to simultaneous activation of both JA- and SA-mediated induced defense (Van Wees *et al.*, 2000).

In line with the results of insect performance assays, at the transcription level, colonization by *Pf.* WCS417r or *Pf.* SS101 as single or mixed cultures led to a similar

extent of down-regulation of transcription of the gene *WRKY70,* which is involved in SA signaling, whereas all treatments up-regulated transcription of the JA marker gene *MYC2,* with colonization by *Pf.* WCS417r showing the highest expression. The gene expression results also indicate that both *Pf.* WCS417r and *Pf.* SS101 modulate plant signaling by suppressing the SA pathway. Van de Mortel et al. (2012) showed that upon root inoculation by *Pf.* SS101, expression of the SA-regulated *WRKY70* gene in the roots is time-dependent, in which the gene is induced during the early stage and suppressed during the later stage of interactions between the plant and the rhizobacterium. The down-regulation of *WRKY70* expression in single or mixed cultures of rhizobacterial colonized plants found in the present study is comparable to expression of the same gene during the later stage of the interactions between roots and *Pf.* SS101 as reported by Van de Mortel *et al.* (2012). In plant interactions with root-colonizing mycorrhizae and the plant growth-promoting fungus (PGPF) *Piriformospora indica*, the fungi induced up-regulation of JA biosynthesis genes in the roots and it has been proposed that the beneficial fungi use the JA-signaling pathway to suppress plant defense (Pozo and Azcon-Aguilar, 2007; Schafer *et al.*, 2009; Zamioudis and Pieterse, 2012). Taken together, we hypothesize that the rhizobacteria *Pf.* WCS417r and *Pf.* SS101 induce up-regulation of JA signaling in plant roots, as is clear from the up-regulation of *MYC2* gene expression, and may involve suppression of the SA-regulated gene *WRKY70* during the later stage of the interactions, a mechanism important to establish rhizobacterial colonization. Moreover, similarities in induction of *MYC2*, *WRKY70* and *NPR1* gene expression in the roots induced by *Pf.* WCS417r or *Pf.* SS101 in single or combined inoculations may indicate that the interactions between the two strains of *P. fluorescens* are direct instead of plant-mediated. Whether the interaction between the two strains is similar in the rhizosphere of wheat plants, from which both strains were originally isolated (Lamers, 1988; De Souza *et al.*, 2003), still needs to be tested.

Using an *in vitro* assay for investigating a microbe-plant-insect interaction, we show that interactions between *Pf.* WCS417r and *Pf.* SS101 in the rhizosphere resulted in different colonization levels of each strain depending on the site of inoculation. Furthermore, different positions of *Pf.* WCS417r and *Pf.* SS101 inoculation singly or combined do not influence induced plant resistance to the insect herbivore *M. brassicae*. It has been proposed that by combining different species or strains of root-associated microbes, ISR is induced via different mechanisms such as the JA and SA pathways and may increase the strength of ISR against plant attackers (Alizadeh *et al.*, 2013; Pangesti *et al.*, 2013). In addition to the issue of mechanisms involved in rhizobacteria-mediated ISR against insect herbivores, results of this study also show that compatibility between

co-inoculated rhizobacteria can potentially be an important factor. Our results support what has been proposed by De Boer *et al.* (1999) using a different biological system, that negative interactions between biocontrol agents can limit the growth of one or both agents below the required threshold; as a result, no enhanced disease suppression occurs. This study shows that competitive interactions between *Pf.* WCS417r and SS101 can interfere with the growth of *Pf.* SS101, thus inhibiting the strain to reach a threshold level needed for ISR to occur. Taken together, compatibility of ISR-inducing agents needs to be first evaluated before considering the different mechanisms involved in induction of ISR, as also suggested in development of reliable biocontrol agents for disease suppression (Raaijmakers *et al.*, 1995b; Janisiewicz, 1996; De Boer *et al.*, 1999). Interestingly, combined inoculation by both strains resulted in lower shoot fresh weight compared to inoculation with *Pf.* WCS417r singly, whereas there was no effect on root fresh weight compared to single inoculation by each strain. Rhizobacterium *Pf.* WCS417r stimulates changes in root architecture via auxin-mediated mechanisms and resulted in increased shoot biomass (Zamioudis *et al.*, 2013). It remains to be investigated via which hormonal pathway *Pf.* SS101 modulates plant growth and if crosstalk between signaling pathways plays a role in reduced shoot fresh weight on the combined-strains treatment compared to *Pf.* WCS417r inoculation singly. Here, the results also indicate that negative crosstalk between JA- and SA-signaling in plant roots may play a role in suppression of plant defense response by different strains of rhizobacteria as mechanism to establish colonization. Although suppression of plant host immunity by beneficial root-associated microbes may also increase the risk of attack by pathogenic microbes, the beneficial microbes may compensate for this by producing antibiotics that reduce the risk of plant invasion by pathogenic microbes. Although the results show that combined inoculation of ISR-inducing agents by two strains of *P. fluorescens* does not affect plant induced defense to aboveground leaf-chewing insect compared to single inoculation, the co-cultivation of the two strains can trigger synthesis of antibacterial compounds that may have positive consequences for protecting plant roots from soil-borne pathogens.

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6

Rhizobacterial colonization of roots modulates plant volatile emission and enhances attraction of a parasitoid wasp to host-infested plants

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ABSTRACT

Beneficial root-associated microbes modify the physiological status of their host plants and affect plant direct and indirect defense to insect herbivores. While the effect of these microbes on direct plant defense to insect herbivores are well described, knowledge on the effect of the microbes on indirect plant defense to insect herbivores is still limited. In this study, we evaluate the role of the rhizobacterium *Pseudomonas fluorescens* WCS417r in indirect plant defense against the generalist leaf-chewing insect *Mamestra brassicae* by combining behavioral, chemical and gene transcriptional approaches. We show that rhizobacterial colonization of *Arabidopsis thaliana* roots results in an increased attraction of the parasitoid *Microplitis mediator* to caterpillar-infested plants. Volatile analysis revealed that rhizobacterial colonization suppressed emission of the terpene (*E*)-α-bergamotene, and the aromatics methyl salicylate and lilial in response to caterpillar feeding. Rhizobacterial colonization decreased the caterpillar-induced transcription of the *Terpene Synthase* genes *TPS03* and *TPS04*. Rhizobacteria enhanced both growth and indirect defense of plants under caterpillar attack. This study shows that rhizobacteria have a high potential to enhance the biocontrol of leaf-chewing herbivores based on enhanced attraction of parasitoids.

Keywords: HIPVs, indirect defense, *Microplitis mediator*, parasitoid behavior, beneficial microbes.

INTRODUCTION

Plants are exposed to attack by various insect herbivores and defend themselves directly, e.g. by producing toxic compounds, and indirectly, e.g. by emitting herbivore-induced plant volatiles (HIPVs) that attract natural enemies of the herbivores (Turlings *et al.*, 1990; Turlings *et al.*, 1995; Dicke and Baldwin, 2010; Clavijo McCormick *et al.*, 2012). The effect of herbivore-induced plant volatiles (HIPVs) on the behavior of natural enemies has been widely studied in the context of interactions between one plant, one insect herbivore, and one natural enemy (Mumm and Dicke, 2010). The exploration of such tritrophic interactions is now being extended to interactions in more complex systems (Dicke *et al.*, 2009; Pineda *et al.*, 2013; Heil, 2014). For instance, insect eggs, multiple insect herbivores, pathogenic and beneficial microbes or belowground herbivores have been shown to affect plant indirect defenses (Rasmann and Turlings, 2007; Soler *et al.*, 2007; Van Dam and Heil, 2011; Fatouros *et al.*, 2012; Reymond, 2013; Zhang *et al.*, 2013; Ponzio *et al.*, 2014). Belowground beneficial microbes such as mycorrhizae, rhizobia, and rhizobacteria constitute a fascinating functional group in the plant-associated community that can enhance plant growth and resistance against pathogens and herbivorous insects (Pozo and Azcon-Aguilar, 2007; Hartley and Gange, 2009; Pineda *et al.*, 2010). However, only more recently the effect of mutualistic microbes on the emission of HIPV and on natural enemies of herbivorous insects has been studied (Schausberger *et al.*, 2012; Pineda *et al.*, 2013).

Root-associated microbes modify plant physiology and, therefore, can have an impact on plant direct and indirect defense against insects. In the context of indirect plant defense, root-colonizing microbes have been shown beneficial for the plant by enhancing the attraction or performance of natural enemies of the herbivores through plant-mediated effects (Gange *et al.*, 2003; Guerrieri *et al.*, 2004; Hempel *et al.*, 2009; Hoffmann *et al.*, 2011; Schausberger *et al.*, 2012). Interestingly, the effect of beneficial microbes on the emission of HIPVs varies, from increased emission of the terpenoids β-ocimene and β- caryophyllene or HIPVs in general (Pineda *et al.*, 2013), to suppressed emission of HIPVs (Fontana *et al.*, 2009). However, experimental evidence showed that increased emission of HIPVs induced by beneficial microbes has differential effects on the attractiveness to the herbivore's natural enemies, from increased attractiveness (Schausberger *et al.*, 2012), to repellence (Pineda *et al.*, 2013).

Synthesis of plant secondary metabolites and HIPVs involved in plant direct and indirect defense is regulated by interconnected phytohormonal signaling pathways.

The plant hormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) are the main phytohormones regulating those herbivore induced responses in the plant (Dicke, 2002; Kessler and Baldwin, 2002; Pieterse *et al.*, 2012). In the context of indirect defense, depending on the species and feeding mode of the insect herbivores, different combinations of hormonal signaling pathways can be induced resulting in the synthesis of specific blends of HIPVs, that attract natural enemies of the herbivores (Zhang *et al.*, 2013; Heil, 2014; Wei *et al.*, 2014). The plant hormone JA regulates the synthesis of VOCs such as Green Leaf Volatiles (GLVs) and terpenoids, whereas SA regulates the shikimate pathway and the emission of volatiles such as methyl salicylate (MeSA) (Dicke, 2002; Van Poecke and Dicke, 2002; Maffei *et al.*, 2011). Several beneficial microbes, such as the well-studied rhizobacterium *Pseudomonas fluorescens* WCS417r, are known to modulate JA and ET signaling (Van Wees *et al.*, 2008; Van der Ent *et al.*, 2009), leading to enhanced expression of defense-associated genes and to modification of the plant's response to insect herbivores from different feeding guilds (Van Oosten *et al.*, 2008; Pineda *et al.*, 2012; Pangesti *et al.*, 2015). In contrast, the effects of root colonization by beneficial microbes on indirect defenses and natural enemies of the herbivores are still largely unknown.

In the present study, we aim to evaluate the role of *P. fluorescens* WCS417r on plant indirect defense upon *Mamestra brassicae* herbivory feeding by evaluating behavioral choices and performance of the parasitic wasp *Microplitis mediator*, a natural enemy of *M. brassicae* caterpillars. This parasitoid is a generalist solitary larval endoparasitoid that parasitizes first to third larval instars of *M. brassicae* (Malcicka and Harvey, 2014). Using the same beneficial microbe and plant combination as in this study, we previously evaluated the emission of plant volatiles upon attack by the phloem feeder *Myzus persicae* and the behavior of the aphid's parasitoid *Diaeretiella rapae* (Pineda *et al.*, 2013). By combining behavioral, chemical and transcriptional approaches we test the hypotheses that rhizobacteria-treated plants will 1) be more attractive to the parasitic wasp *M. mediator* upon caterpillar herbivory and support better performance of the parasitoid; 2) emit higher amounts of VOCs upon caterpillar herbivory; 3) increase the expression of the *Terpene Synthase* genes *TPS03*, encoding (*E,E*)-α-farnesene synthase, an enzyme involved in biosynthesis of (*E,E*)-α-farnesene (Huang *et al.*, 2010) and *TPS04*, encoding geranyllinalool synthase (GES), a major enzyme involved in 4,8,12-trimethyltrideca-1,3,7,11-tetraene ((*E,E*)-TMTT) biosynthesis (Herde *et al.*, 2008) upon feeding by caterpillar herbivory.

MATERIALS AND METHODS

Plant growth and insect rearings

Seeds of *Arabidopsis thaliana* Col-0 were sown in sand (Masonry sand, Van Leusden B.V., The Netherlands). Seedlings (10-day-old) were transplanted into pots (120 ml) containing a 1:1 (v/v) mixture of potting soil:sand. In this study, we used commercial potting soil for *Arabidopsis* (Lentse *Arabidopsis*-grond, Lent, The Netherlands). Plant growth conditions have been described in Pangesti *et al.* (2015). Once a week, 10 ml of a half-strength Hoagland solution/pot (Sigma-Aldrich) was added (Van Oosten *et al.*, 2008). In all experiments, 5-to-6-week old plants in the vegetative stage were used.

The generalist insect herbivore *M. brassicae* L. (Lepidoptera: Noctuidae; Cabbage moth) was reared on *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) in a climate chamber (22 ± 2 °C, 40 - 50 % RH, 16:8 h photo:scotophase). Neonate larvae were used in the experiments. The solitary parasitoid *M. mediator* (Hymenoptera: Braconidae) was reared on *M. brassicae* feeding on Brussels sprouts in a greenhouse (22 ± 1 °C, 60 ± 10 % RH, 16:8 h photo:scotophase). Parasitoid cocoons were collected and incubated until emergence in a climate cabinet (22 °C, 16:8 h photo:scotophase), supplemented with honey and water. In all experiments, 2-to-7-days old naive mated female parasitoids were used.

Rhizobacterium *Pf.* WCS417r growth, inoculation of soil media and quantification

A rifampicin-resistant, non-pathogenic rhizobacterium strain *Pf.* WCS417r was used in this study. The rhizobacterium was grown for 48 h at 28°C on King's B (KB) medium agar plates containing rifampicin (25 μg ml^{-1}) (Pieterse *et al.*, 1996). Prior to mixing with sterile soil, bacterial cells were collected, resuspended in 10 mM $MgSO_4$, and washed three times with 10 mM $MgSO_4$. Afterwards, the bacterial cells were re-suspended in 10 mM $MgSO_4$ and adjusted to a cell density of 1 x 10^9 cfu ml^{-1} (OD660 = 1.0). For rhizobacterial treatment, 50 ml of the bacterial suspension was mixed per kg of autoclaved soil; for control treatment, 50 ml of 10 mM $MgSO_4$ was mixed per kg of sterile soil. Quantification of *Pf.* WCS417r in *A. thaliana* roots was done for each batch following a well-established method (Pieterse *et al.*, 1998; Pangesti *et al.*, 2015).

Behavioral test of the parasitoid wasp *M. mediator*

Dual-choice tests were performed using a closed-system Y-tube olfactometer, that was illuminated from above (Snoeren *et al*., 2009; Pineda *et al*., 2013). Details of the Y-tube olfactometer set up and behavioral tests were similar to the description in Pineda *et al*. (2013). Experiments were repeated on several days, with ca. 20 female wasps tested per pair-wise comparison per day. In total, 4-5 sets of plants and 88-98 female wasps were evaluated per pair-wise comparison. As odor sources, plants were tested that had been subjected to one of four treatments, based on the presence/absence of rhizobacteria and *M. brassicae* caterpillars: 1) control uninfested (C); 2) rhizobacteria-treated uninfested (R); 3) control infested with *Mamestra* caterpillars (CM); 4) rhizobacteria-treated plants infested with *Mamestra* caterpillars (RM). In the treatments with caterpillars, *A. thaliana* plants (5 - 6 weeks old) were infested with three neonate larvae of *M. brassicae* during 3 days before the experiments. Individual plants from all treatments were confined in a plastic container (height 14 cm; upper diameter 11 cm, lower diameter 8.5 cm), covered with insect-proof mesh cloth and sealed with elastic bands. Plants were kept in a growth chamber under 16:8 h photo:scotophase (200 μmol m^{-2} s^{-1}) at 21 ± 1°C and 60 - 70 % RH. Four plants together comprised an odor source. After the behavioral bioassay, *M. brassicae* larvae from control (CM) and rhizobacteria-treated plants (RM), were recovered and weighed (microbalance CP2P, Sartorius AG, Germany). Additionally, a pool of four plant rosettes of all treatments (C, R, CM, RM) were weighed after each dual-choice assay.

To evaluate if *M. mediator* responds to volatiles from caterpillar-infested *A. thaliana* Col-0 as host-location cues, the following dual-choice experiment was conducted as a control: control uninfested (C) versus control caterpillar-infested plants (CM). To assess whether *M. mediator* responds to volatiles from rhizobacteria-treated caterpillar-infested plants, the following experiment was conducted: rhizobacteria-treated undamaged (R) versus rhizobacteria-treated caterpillar-infested plants (RM). To test the second hypothesis, that the effect of rhizobacteria is a result of the plant's interaction with both *M. brassicae* and rhizobacteria and not simply a rhizobacteria-plant interaction, the following experiments were conducted: control caterpillar-infested (CM) versus rhizobacteria-treated caterpillar-infested plants (RM); control uninfested (C) versus rhizobacteria-treated uninfested (R).

Headspace collection and analysis of volatiles

To link parasitoid behavior to volatile emission, collection of plant volatiles was conducted simultaneously with the behavioral assays. In order to correct for background volatiles, collection of volatiles from empty jars, empty plant pots, plant pots filled with autoclaved soil, and plant pots filled with autoclaved and rhizobacteria-treated soil were sampled as well. Collection of plant volatiles was done for 4 h by drawing air out of the jars at a rate of 200 mL min^{-1} with the help of an external pump through a stainless steel cartridge (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) (Pineda *et al.*, 2013). Immediately after the collection of volatiles, plant rosettes were weighed and the Tenax TA cartridges were dry-purged for 10 min with nitrogen (N2, 50 mL min^{-1}) at room temparature (RT) and then also stored at RT until analysis. For each treatment, 9 to 10 replicates were sampled.

Headspace samples were analysed with a Thermo Trace Ultra gas chromatography (GC) coupled to a Thermo Trace DSQ quadruple mass spectrometer (MS) both from Thermo (Thermo Fisher Scientific, Waltham, MA, USA). Volatiles were desorbed from the cartridges using a thermal desorption system at 250°C for 10 min (Ultra 50:50, Markes) with a helium flow of 20 mL min^{-1}. Analytes were focused at 0 °C on an electronically cooled sorbent trap (Unity, Markes) and were then transferred in splitless mode to the analytical column (ZB-5MSi, 30 m x 0.25 mm i.d. x 0.25 mm film thickness with 5 m built-in guard column (Phenomenex, Torrence, CA, USA) situated in the GC oven for further separation by rapid heating of the cold trap at a rate of 40 °C s^{-1} to 280 °C, which was maintained for 10 min. The GC was held at an initial temperature of 40 °C for 2 min followed by a linear thermal gradient of 10 °C min^{-1} to 280 °C and held for 4 min under a column flow of 1 mL min^{-1}. The column effluent was ionized by electron impact ionisation at 70 eV. Mass spectra were acquired by scanning from 35–350 m/z at a scan rate of 5.38 scans s^{-1}. MS transfer line and ion source were set to 275 and 250 °C, respectively. Tentative identification of compounds was made by comparison of mass spectra with those in NIST 2005 and the Wageningen Mass Spectral Libraries of Natural Products. Experimentally calculated linear retention indices (LRI) were also used as additional criterion to identify the compounds. Relative quantification (peak areas of individual compounds) was obtained using a single (target) ion, in selected ion monitoring (SIM) mode. The individual peak areas of each compound were further used in the statistical analysis. Volatiles from empty glass jars, empty plant plastic pots, pots filled with autoclaved soil, pots filled with autoclaved and rhizobacteria-treated soil, clean Tenax TA and the analytical instrument itself were used as a control measure for artefacts.

Gene transcript analyses of two *Terpene Synthase* Genes, *TPS03* and *TPS04*

To evaluate if rhizobacterial colonization itself and in combination with insect herbivory by *M. brassicae* has an effect on the transcription of two genes coding for enzymes involved in plant volatile producion, the *Terpene Synthase* genes *TPS03* and *TPS04*, the same four treatments as in the behavioral assay were arranged. Fully expanded leaves of plants exposed to feeding larvae were sampled after gently removing the caterpillars. Leaves were harvested at 10 and 24 h after insect infestation (hpi). Leaves of uninfested plants were treated and harvested at similar time points as those of infested plants. Leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C for RNA extraction. For each treatment, five biological replicates were used, each consisting of six to nine local leaves pooled from three individual plants. The procedure for processing leaf samples, measurement of RNA quality and procedure of cDNA synthesis followed the methods described in Pangesti *et al.* (2015).

Transcripts of the *Terpene Synthase* (*TPS*) genes *TPS03* (AT4G16740) with sequences of primers forward: 5'-GCCACCATCCTCCGTCTC-3' and reverse: 5'-CCAAGCCACACCGATAATTCC-3', *TPS04* (AT1G61120) with sequences of primers forward: 5'-TCGCAGCACACACCATTG-3' and reverse: 5'-GAGCAGCACGGAGTTCATC-3' (Snoeren *et al.*, 2010) were quantified in a qRT-PCR (CFX96™ Real-Time System, BIO-RAD, Hercules, CA, USA). Efficiency of each primer was determined before qRT-PCR analysis. Thermal cycling conditions consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 45 s. For each primer pair, controls without addition of template were performed to confirm that primer dimers were not interfering with detection of amplification. The transcript level for each tested gene was calculated relative to the reference genes *ELONGATION FACTOR 1α (EF1α)* (AT5G60390) with sequences of primers forward: 5'-TGAGCACGCTCTTCTTGCTTTCA-3' and reverse: 5'-GGTGGTGGCATCCATCTTGTTACA-3' and *F-BOX FAMILY PROTEIN (FBOX)* (AT5G15710) with sequences of primers forwards: 5'-TTTCGGCTGAGAGGTTCGAGT-3' and reverse: 5'-GATTCCAAGACGTAAAGCAGATCAA-3' (Remans *et al.*, 2008) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Performance of the parasitoid *M. mediator*

To evaluate the effect of rhizobacteria on the development of *M. mediator*, performance of the parasitoid in its host *M. brassicae* feeding on either control plants

(CM) or rhizobacteria-treated plants (RM) was assessed. Neonate caterpillars were allowed to feed for 3 days on control plants (C) or rhizobacteriatreated plants (R). One parasitoid female was allowed to parasitize 3 caterpillars reared on control plants (C) and 3 caterpillars reared on rhizobacteria-treated plants (R). Afterwards, these parasitized caterpillars were placed again on plants subjected to the corresponding treatments. All plants were placed individually in plastic containers under the same conditions as described above. Plants were watered 3 times a week adding a total of 60 mL of water. On day 8 after infestation, the parasitized caterpillars were transferred to a second plant to avoid food limitation. Survival of parasitized caterpillars and fresh plant biomass were assessed after 8 days of feeding by parasitized caterpillars. Once cocoons were formed, each cocoon was individually kept in a glass tube closed with cotton wool until adult wasps emerged. Cocoon fresh weight was measured 2 days after their formation. Once the adult parasitoids emerged, their sex was recorded and then the parasitoids were anesthetized using CO_2 and weighed on a microbalance to the nearest µg. The following parameters of parasitoid performance were measured: development time from parasitization to cocoon formation; time from cocoon formation to adult emergence (pupal development time), and time from parasitization to adult emergence (total development time); cocoon fresh weight; larval, pupal, and overall survival; fresh weight of male and female parasitoids. In total 25 plants and 75 parasitized larvae were assessed for each treatment.

Statistics

Behavioral data were analyzed using a binomial test. After each of the behavioral assays, we assessed the performance of *M. brassicae* on the plants used for the behavioral assays. Data of *M. brassicae* larval weight were analyzed with a Linear Mixed Model (LMM) with treatments as fixed factor and experimental group (since four plants were placed in the glass jar and larvae would move around during the behavioral test) as random factor. Data of plant shoot biomass comparing four treatments were analyzed with a two-way ANOVA. Plant volatile data were log-transformed, univariate scaled and analyzed with multivariate Projection to Latent Structures-Discrimination Analysis (PLS-DA) (SIMCA P+12.0, Umetrics AB, Umeå, Sweden). Pair-wise comparisons between treatments of the quantity emitted of each volatile compound were performed with a t-test. Gene transcription data were log-transformed and analyzed with a two-way ANOVA with treatment and time as factors.

Developmental times of *M. mediator* were analyzed using a Generalized Linear Mixed Model (GLMM) with treatment as a fixed factor and plant number as random

factor. The survival data were analyzed using a Generalized Linear Model (GLM) with binomial distribution and logit link function. Adult fresh weight was analyzed with a t-test. LSD tests were used for post-hoc comparisons when necessary. All data, except the volatile data, were analyzed using GenStat 16th edition, VSN International Ltd.

RESULTS

Rhizobacterial colonization enhances attraction of the parasitoid *M. mediator* in plants infested with *M. brassicae*

In dual-choice olfactometer assays, the parasitoid wasps did not discriminate between volatiles emitted from control plants (C) and those emitted from rhizobacteria-treated plants (R) (Fig. 1; binomial test, $P = 0.918$). In contrast, the wasps significantly preferred the volatiles emitted from *M. brassicae*-infested plants (CM) over volatiles emitted from control plants (C) (binomial test, $P < 0.001$). Likewise, the parasitoid wasp also significantly preferred the volatiles emitted from rhizobacteria-treated plants infested with *M. brassicae* (RM) over volatiles emitted from rhizobacteria-treated plants (R) (binomial test, $P < 0.001$). Interestingly, rhizobacterial colonization of *A. thaliana* roots significantly increased the parasitoid wasp preference towards the volatiles emitted from *M. brassicae*-infested plants (RM) compared to the volatiles emitted from plants without rhizobacteria and infested with *M. brassicae* (CM) (binomial test, $P = 0.033$).

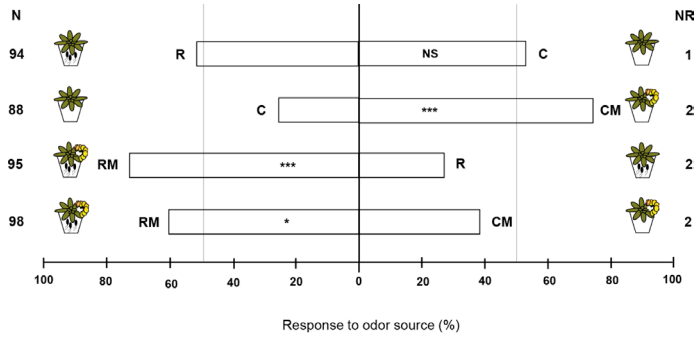

Figure 1. Response of *M. mediator* naive female parasitoids in a Y-tube olfactometer to the volatiles of *A. thaliana* Col-0 plants from different treatments. Treatments were control plants (C), rhizobacteriatreated plants (R), control plants infested with *M. brassicae* caterpillars (CM), or rhizobacteria-treated plants infested with *M. brassicae* caterpillars (RM). Nine to ten sets of plants were used per pair-wise comparison, each consisting of four plants per treatment. For treatments with *M. brassicae* (CM/RM), three neonate larvae were inoculated per plant and incubated for 3 days before the test; parasitoids used for the test were 2-7 days old. Bars represent percentage of parasitoids choosing each of the two odor sources; numbers on the left (N) represent total number of parasitoids choosing either of the odor sources; numbers on the right (NR) represent the number of non-responsive parasitoids. Asterisks indicate significant differences (binomial test: *, $P < 0.05$; ***, $P < 0.001$; NS, not significant).

The rhizobacterial treatment resulted in increased larval weight at 3 dpi (Fig. 2A; *df* = 1, 81.9; *F* = 4.29; *P* = 0.042). Assessing plant shoot fresh weight after 6 days of *M. brassicae* feeding showed that both *M. brassicae* feeding and rhizobacterial colonization significantly affected plant biomass, but there was no interaction between the two factors (two-way ANOVA, *Mamestra*: *df* = 1, 53; F = 31.76; *P* < 0.001; Rhizobacteria: *df* = 1, 53; *F* = 4.12; *P* = 0.048; *Mamestra**Rhizobacteria: *df* = 1, 53; *F* = 0.79; *P* = 0.379) (Fig. 2B). Rhizobacterial colonization of *A. thaliana* Col-0 roots (R) had no effect on the shoot fresh weight in comparison to control plants (C). Interestingly, under caterpillar attack, rhizobacteria-treated plants (RM) grew better compared to control plants infested with caterpillars (CM). Whereas plant shoot fresh weight after 3 days of *M. brassicae* feeding showed that herbivory significantly affected plant biomass, rhizobacteria had no effect on plant biomass (Supplementary Materials Fig. S1; two-way ANOVA, *Mamestra*: *df* = 1, 37; *F* = 5.75; *P* = 0.022; Rhizobacteria: *df* = 1, 37; *F* = 1.72; *P* = 0.199; *Mamestra**Rhizobacteria: *df* = 1, 37; *F* = 0.41; *P* = 0.525). In the rhizosphere of control plants no rifampicin-resistant rhizobacteria were detected (detection limit 10^2 cfu/g), whereas in the rhizosphere of rhizobacteria-treated plants the numbers of rhizobacteria were overall above 10^5 cfu.g^{-1} roots (Supplementary Materials table S1).

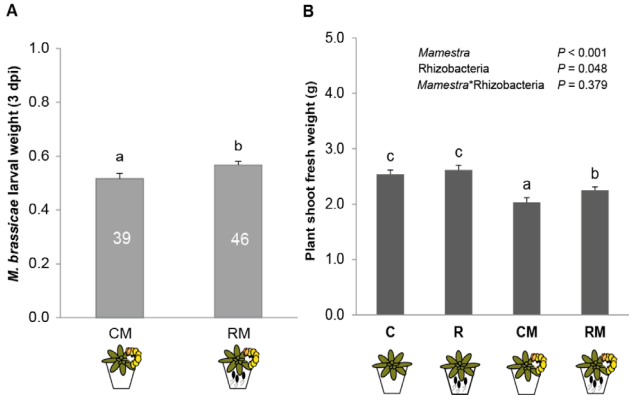

Figure 2. Caterpillar body mass as measured for caterpillars feeding on the sets of plants used during two-choice Y tube olfactometer assays. (A) Larval weight of *M. brassicae* caterpillars on control plants (CM) and rhizobacteria-treated plants (RM) measured after 3 days post infestation (dpi). Three neonate larvae were placed on each plant and a set of four plants was used in each experiment. Numbers inside each bar represent the number of larvae surviving on the day of weight assessment. Data shown are means (± SE) of larval weight. Different letters above bars indicate significant differences between treatments (LMM, *P* < 0.05, LSD test). (B) Plant shoot fresh weight of control plants (C), rhizobacteria-treated plants (R), control plants infested with *Mamestra brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM) at 6 days post infestation of the herbivore (two-way ANOVA, *N* = 12 – 15 plants, *P* < 0.05, LSD test). Data shown are means (± SE). Different letters above bars indicate significant difference between treatments (*P* < 0.05).

Herbivory by *M. brassicae* increases volatile emission of control and rhizobacteria treated *A. thaliana* plants

A PLS-DA analysis of control plants (C) and control plants infested with *M. brassicae* (CM) showed four significant principal components (PC) with the first two explaining 32.14 and 14.94 % of the total variance, respectively (Fig. 3A). The first component (PLS 1) separated the volatile blends based on the presence or absence of *M. brassicae* caterpillars. In the headspace, a total of 13 compounds were detected, and 3 compounds had a VIP value higher than 1(Supplementary materials Table S2). VIP values indicate the importance of the variable, i.e. the volatile compound, in the projection and those with values larger than 1 are the most influential for the model (Eriksson *et al.*, 2006). In decreasing order of importance, these compounds were methyl salicylate, (*E,E*)-TMTT, and methyl *cis*-dihydrojasmonate (Fig. 3B). These three compounds were emitted in significantly higher amounts by CM than by C plants (Supplementary materials Table S3; t-test; $P < 0.05$). All other compounds were emitted in statistically similar quantities from CM and C plants (Supplementary materials Table S3; t-test; $P > 0.05$).

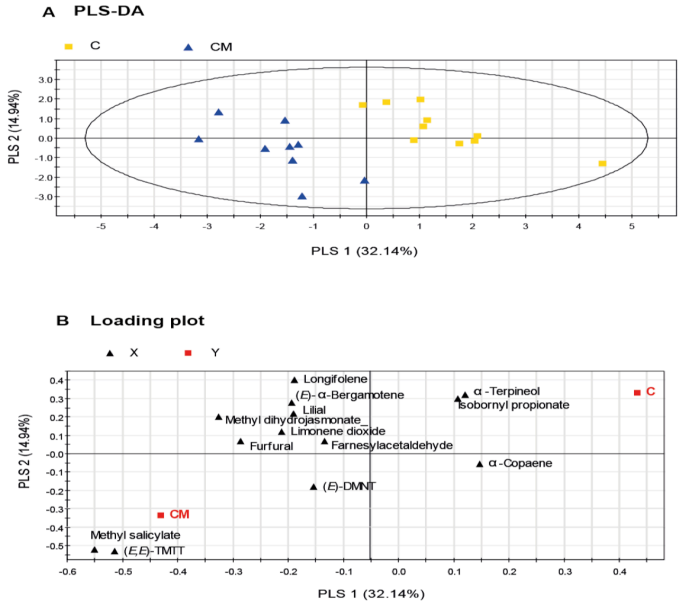

Figure 3. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparing *A. thaliana* Col-0 volatile blends from control plants (C) versus control plants infested with *Mamestra brassicae* (CM) for 3 days before volatile collection. (A) Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample ($N = 9 – 10$ replicates). (B) Loading plot of the first two components of the PLS-DA, showing contribution of each volatile compound to the separation of the two treatments.

A PLS-DA analysis of rhizobacteria-treated plants (R) and rhizobacteria-treated plants infested with *M. brassicae* (RM) showed one significant PC explaining 32.24 % of the total variance (Fig. 4A). The second axis is shown for representational purposes. This PC also separated the volatile blends based on the presence or absence of caterpillar *M. brassicae*. Of the 13 compounds recorded, 3 compounds showed a VIP value higher than 1 (Supplementary materials Table S2). In decreasing order of importance, these compounds were (*E,E*)-TMTT, methyl *cis*-dihydrojasmonate, α-terpineol (Fig. 4B). (*E,E*)-TMTT and (*E*)-α-bergamotene were emitted in significantly higher amounts by RM than by R plants (Supplementary materials Table S3; t-test; $P < 0.05$), whereas the emission rates of all other compounds were statistically similar in RM and R plants.

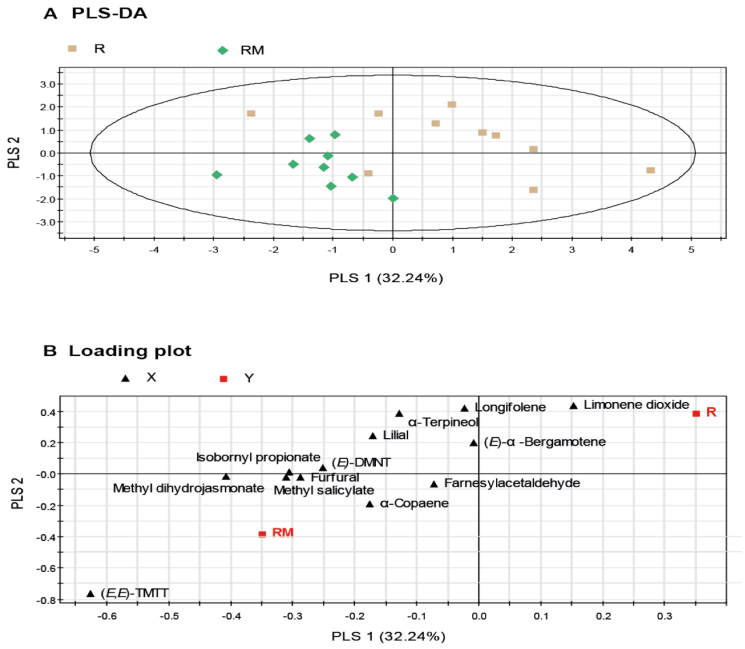

Figure 4. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparing *A. thaliana* Col-0 volatile blends from rhizobacteria-treated (R) versus rhizobacteria-treated plants infested with *M. brassicae* (RM) for 3 days before volatile collection. (A) Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample (N = 9 – 10 replicates). (B) Loading plot of the first two components of the PLS-DA, showing contribution of each volatile compound to the separation of the two treatments.

When all four treatments of control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM) and rhizobacteria-treated plants infested with *M. brassicae* (RM) were analyzed together in one PLS-DA analysis, it gave one significant PC explaining 26.09 % of the total variance. Similar to the PLS-DA comparing C-CM and R-RM, this PC separated the volatile blends based on the presence or absence of caterpillar *M. brassicae*. Among the 13 compounds recorded, 4 compounds had VIP values higher than 1. In decreasing order of importance, these compounds were (*E,E*)-TMTT, methyl salicylate, methyl *cis*-dihydrojasmonate and lilial (Supplementary materials Fig. S2).

Rhizobacterial colonization suppresses volatile emission of *A. thaliana* following *M. brassicae* herbivory

A PLS-DA comparing volatiles emitted by control plants infested with *M. brassicae* (CM) and rhizobacteria-treated plants infested with *M. brassicae* (RM) showed one significant PC explaining 22.73 % of the total variance (Fig. 5A). The second axis is shown for representational purposes. This PC separated the volatiles based on the presence or absence of the rhizobacteria. Five compounds had a VIP value higher than 1 (Supplementary materials Table S2) and these were methyl salicylate, (*E*)-α-bergamotene, lilial, longifolene, methyl cis-dihydrojasmonate, in decreasing order of importance (Fig. 5B). Among the five compounds, methyl salicylate, (*E*)-α-bergamotene and lilial were emitted in significantly lower amounts by RM than by CM plants (Supplementary materials Table S3; t-test; $P < 0.05$), whereas emission rates of all other compounds were statistically similar in RM and CM plants. Interestingly, a PLS-DA comparing volatiles of control (C) and rhizobacteria-treated plants (R) showed no significant principal component. Pair-wise comparisons for the quantities emitted of each of the 13 compounds detected in the two treatments did not show differences (Supplementary materials Table S3; t-test; $P > 0.05$).

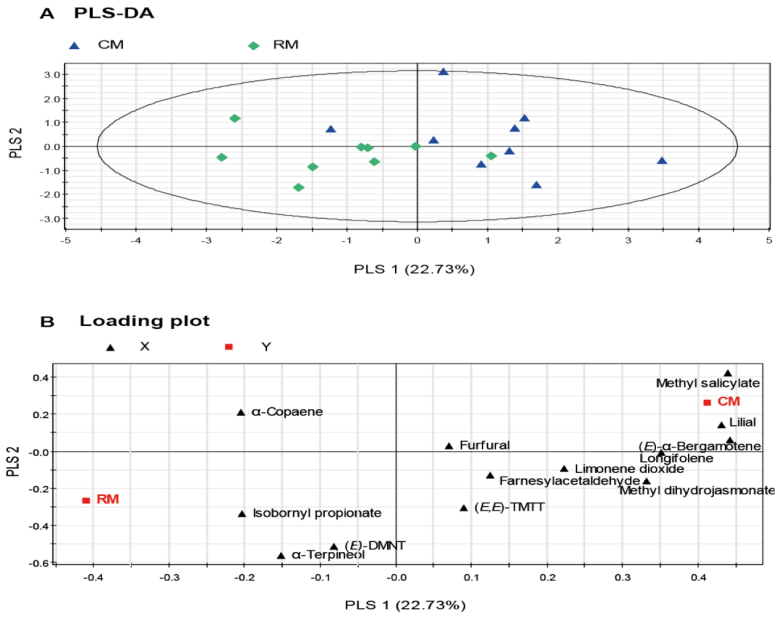

Figure 5. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparing *A. thaliana* Col-0 volatile blends from control plants infested with *M. brassicae* (CM) versus rhizobacteria treated plants infested with *M. brassicae* (RM) for 3 days before volatile collection. (A) Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample ($N = 9 – 10$ replicates). (B) Loading plot of the first two components of the PLS-DA, showing contribution of each volatile compound to the separation of the two treatments.

Rhizobacterial colonization modifies transcription of terpene synthase genes *TPS03* and *TPS04*

Transcript analyses of *TPS03* and *TPS04* showed that these genes were affected by both treatment and time (two-way ANOVA, effect of treatment on *TPS03* expression: $df = 3, 32$; $F = 63.11$, $P < 0.001$; time: $df = 1, 32$; $F = 8.44$, $P < 0.007$; effect of treatment on *TPS04* expression: $df = 3, 32$; $F = 113.96$, $P < 0.001$; time: $df = 1, 32$; $F = 4.68$, $P = 0.038$). However, there was no interaction between treatment and time for either *TPS03* or *TPS04* expression.

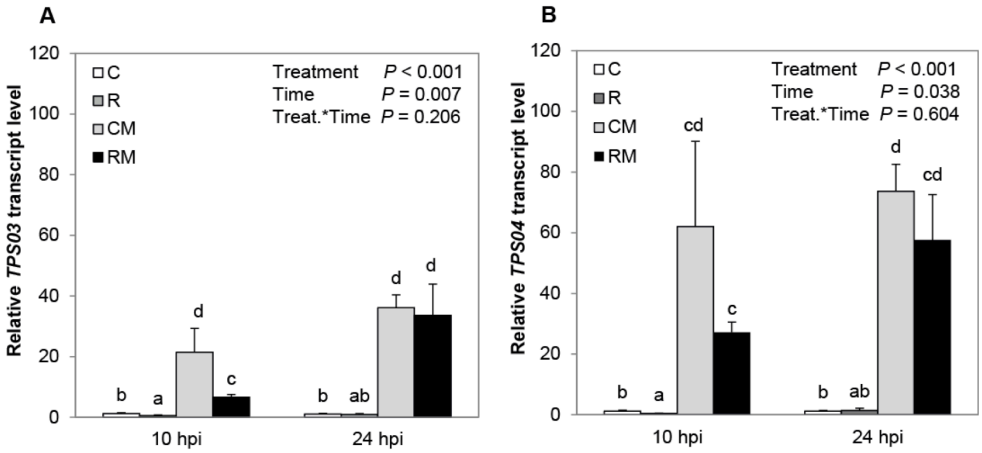

Figure 6. Relative transcript levels of *TPS03* and *TPS04* in local leaves of *A. thaliana* Col-0 control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), or rhizobacteria-treated plants infested with *M. brassicae* (RM) for 10 and 24 h post infestation (hpi). Three neonate *M. brassicae* larvae were inoculated for treatments CM and RM. Transcript levels (mean ± SE) of tested genes which were normalised relative to reference genes *EF1α* and *FBOX*, and measured relative to the control plants ($N = 5$ replicates, each from a pool of 3 plants). Different letters above bars indicate significant differences between treatments (two-way ANOVA, $P < 0.05$, LSD test).

At 10 hpi, rhizobacterial colonization (R) resulted in a down-regulation of both *TPS03* and *TPS04* transcription in comparison to control plants (C) (Fig. 6A, 6B); however, at 24 hpi, gene expression did not differ between treatments (two-way ANOVA, $P > 0.05$, LSD test). At both time points, feeding damage of *M. brassicae* (CM) resulted in a significantly increased expression of *TPS03* and *TPS04* in comparison to control plants (C). Rhizobacteria-treated plants infested with *M. brassicae* (RM) resulted in significantly higher expression of *TPS03* and *TPS04* when compared to uninfested plants (R). Contrary to our hypothesis, at 10 hpi the expression of *TPS03* in rhizobacteria-treated plants infested with *M. brassicae* (RM) was significantly lower than in control plants infested with *M. brassicae* (CM). A lower mean expression level was also found for *TPS04*; however, the difference was not significant. Interestingly, after 24 h feeding by *M. brassicae* on rhizobacteria-treated plants (RM), the expression of *TPS03* significantly increased to a similar level as for control plants infested with *M. brassicae* (CM). A similar pattern was also observed in the expression of *TPS04*.

Rhizobacterial colonization does not affect the performance of the parasitoid *Microplitis mediator*

Rhizobacterial colonization had no effect on performance parameters of the parasitoid *M. mediator* (Fig. 7A): developmental time of egg/larva (GLMM; *df* = 1, 48.2; Wald stat. = 2.41; *P* = 0.127), pupa (GLMM; *df* = 1, 148; Wald stat. = 0.21; *P* = 0.65;) and egg – pupa (GLMM; *df* = 1, 48; Wald stat. = 1.25; *P* = 0.27). Rhizobacterial colonization had no effect on survival of egg/larva (GLM, *df* = 1, 49; Wald stat. = 0.29; *P* = 0.593), pupa (GLM, *df* = 1, 43; Wald stat. = 0.07 *P* = 0.789) and survival during development from egg to pupa (GLM, *df* = 1, 48; Wald stat. = 0.39; *P* = 0.534) (Fig. 7B). Rhizobacterial treatment also did not affect *M. mediator* fresh weight of adult males (t-test, *df* = 1, *F* = 0.023, *P* = 0.964) or females (t-test *df* = 1, *F* = 0.017, *P* = 0.904) (Fig. 7C).

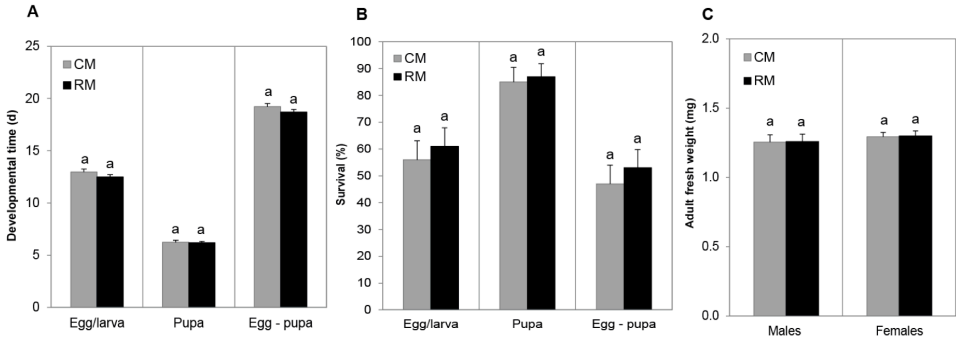

Figure 7. Performance parameters of the parasitoid wasp *M. mediator* developing in its host *M. brassicae* feeding on either control (CM) or rhizobacteria-treated (RM) *A. thaliana* Col-0 plants. Three parasitized larvae were placed on each plant (*N* = 25 plants). Effect of rhizobacterial colonization on (A) developmental time of parasitoid egg/larva; pupa; egg - pupa (GLMM), (B) survival of parasitoid egg/larva; pupa; egg - pupa (GLMM) and (C) fresh weight of adult parasitoid males and females (t-test). Different letters above bars indicate significant difference between treatments (*P* < 0.05).

DISCUSSION

Our study shows that *P. fluorescens* WCS417r colonization of *A. thaliana* roots results in an increased attraction of the parasitoid wasp *M. mediator* to host-infested plants. In the absence of the herbivore, *M. mediator* did not discriminate between rhizobacteria-treated plants and control plants, indicating that rhizobacteria themselves did not emit or induce volatiles that affect the searching behavior of the parasitoid. Previous studies likewise found that different groups of beneficial microbes in general, positively affect

the attraction of arthropod natural enemies in different plant species (Guerrieri *et al.*, 2004; Schausberger *et al.*, 2012; Battaglia *et al.*, 2013). For instance, inoculation of bean plants (*Phaseolus vulgaris* L.) with the mycorrhiza *Glomus mosseae* changed the composition of HIPVs after spider-mite attack and increased the attraction of predatory mites to the spider mite-infested plants (Schausberger *et al.*, 2012). Similarly, tomato plants colonized by the root-associated fungus *Trichoderma longobrachiatum* are more attractive to the aphid parasitoid *Aphidius ervi* (Battaglia *et al.*, 2013). In contrast, a recent study using the same rhizobacteria and plant combination as in the present study demonstrated that the parasitoid *Diaeretiella rapae* was less attracted to rhizobacteria-inoculated plants infested by its host, the aphid *Myzus persicae*, than to host-infested plants without rhizobacteria (Pineda *et al.*, 2013). The effect of mycorrhizal fungi on herbivore parasitization depended on the mycorrhizal species (Gange *et al.*, 2003). Our present study, together with Pineda *et al.* (2013) shows that the effect of a certain beneficial microbe on indirect plant defense depends on the species of insect herbivores and their parasitoids.

Volatile analysis of control- and rhizobacteria-treated plants infested with *M. brassicae* (CM versus RM) showed that both treatments resulted in emission of the same compounds, but different quantities of longifolene, (*E*)-α-bergamotene, methyl salicylate, lilial, and methyl *cis*-dihydrojasmonate. In contrast to our hypothesis, instead of an increased emission of HIPVs, rhizobacterial colonization of *A. thaliana* roots reduced the emission of several HIPVs. Quantitative analysis showed that the rhizobacteria-treated plants infested with caterpillars (RM) emitted lower amounts of the aromatics methyl salicylate, lilial and the terpene (*E*)-α-bergamotene in comparison to control plants infested with caterpillars (CM), even though the total emission of volatile compounds was similar for both treatments. In line with this finding, a previous study on *Plantago lanceolata* plants found that mycorrhizal (*Rhizophagus irregularis*, formerly known as *Glomus intraradices*) colonization suppressed the emission of several terpenoids by plants infested with the caterpillar *Spodoptera exigua* (Fontana *et al.*, 2009). Whether mycorrhiza-induced suppression of HIPVs affects the behavior of natural enemies of herbivorous insects has not been evaluated, since most studies have focused either on the emission of VOCs or on the effects on the parasitoids. Showing the opposite pattern, in our previous study with rhizobacteria and aphids, the decreased attraction of aphid parasitoids to rhizobacteria-treated host-infested plants was associated with an increased emission of HIPVs (Pineda *et al.*, 2013). These examples indicate that root-associated microbe-induced emission of HIPVs does not necessarily translate to

increased attractiveness of natural enemies of herbivores. The blend composition likely plays an important role (Van Wijk *et al.*, 2011).

Transcriptional analysis of the *Terpene Synthase* genes *TPS03* and *TPS04* showed that rhizobacterial colonization reduced the expression of those genes in plants infested with *M. brassicae*. Overall, the gene transcriptional results correlate with volatile analysis showing that in caterpillar-infested plants, rhizobacterial colonization reduced the emission of the terpene (*E*)-α-bergamotene. In *A. thaliana* Col-0, *TPS03* encodes an enzyme that regulates the biosynthesis of a sesquiterpene, (*E,E*)-α-farnesene (Huang *et al.*, 2010), and *TPS04* encodes geranyllinalool synthase (GES), a major enzyme involved in biosynthesis of the homoterpene (*E,E*)-TMTT (Herde *et al.*, 2008). The results show that the expression of *TPS04* was also higher in caterpillar-infested plants of both control- or rhizobacteria-treated plants (CM/RM) compared to uninfested plants (C/R), however, the expression did not differ between CM and RM. The *TPS04* gene expression results are in line with the volatile results showing an increased emission of (*E,E*)-TMTT in both control- and rhizobacteria-treated plants infested with *M. brassicae* (CM, RM) compared to uninfested treatments (C/R), but quantitatively there is no difference in the amount of (*E,E*)-TMTT in CM and RM. The expression of *TPS03* was also significantly higher in caterpillar-infested plants of both control- or rhizobacteria-treated plants (CM/RM) compared to uninfested plants (C/R), nevertheless the sesquiterpene (*E,E*)-α-farnesene was not detected in the volatile blend of any of the treatments. Potential explanations could be that the quantity of the compound was below the detection threshold. Similar as for *TPS03*, the results of gene transcription and volatile analysis are not supporting our hypothesis that the enhanced expression of JA-regulated genes by the rhizobacterium *P. fluorescens* WCS417r induced by caterpillar herbivory (Van Oosten *et al.*, 2008; Pangesti *et al.*, 2015) would positively correlate with the biosynthesis of HIPVs, that is known to be regulated by JA signaling (Dicke *et al.*, 2009; Dicke and Baldwin, 2010). The mechanism underlying the difference between the induction of marker genes involved in plant direct and indirect defense under caterpillar attack remains to be investigated.

Plants face an important dilemma, i.e. whether to allocate resources to growth or defense (Herms and Mattson, 1992) and it is likely that the interaction with belowground beneficial microbes could help plants to accommodate both strategies (Bennett *et al.*, 2006; Pangesti *et al.*, 2013). Our data show that rhizobacterial colonization resulted in an increase in plant growth in the presence of caterpillars but no increased growth of uninfested plants, and this effect is only significant after longer caterpillar infestation.

The rhizobacterium *P. fluorescens* WCS417r is known to promote root growth by modulating auxin signaling in the plant (Zamioudis *et al.*, 2013), and therefore will increase plant access to soil nutrients and enhance plant growth. A study on cotton plants (*Gossypium hirsutum* L.) showed that increased nitrogen fertilization suppressed the synthesis of various terpenoids by S. *exigua*-induced plants (Chen *et al.*, 2008). We hypothesize that in our study system *P. fluorescens* WCS417r led to an increase in the levels of nitrogen or other mineral nutrients in the plants, thus suppressing the emission of terpenoids and aromatic volatiles following caterpillar herbivory. The synthesis of volatile terpenoids is regulated by the JA signaling pathway (Dicke, 2002; Van Poecke and Dicke, 2002; Maffei *et al.*, 2011), and JA is also the main plant hormone regulating the switch from growth to defense (Bennett *et al.*, 2006; Pangesti *et al.*, 2013). As shown in this study, rhizobacteria also play an important role in plant defense and growth, but whether JA signaling regulates a rhizobacteria-induced trade-off between growth and synthesis of HIPVs requires further research. Taken together, these results may imply that rhizobacterial colonization can relieve plant resource allocation to invest in both growth and indirect defense to increase fitness during caterpillar attack.

This study shows that rhizobacterial colonization of plant roots results in an increase in larval weight of the herbivore, but the positive effect on the herbivore was compensated by increased attraction of the parasitoid *M. mediator* to caterpillar-infested plants. We previously reported that the effect of rhizobacteria on plant direct defense against *M. brassicae* was variable and that soil nutrient level influenced the strength of this direct defense (Pangesti *et al.*, 2015). Interestingly, when we incorporate a natural enemy of the herbivore in the study system, the effect of rhizobacteria on plant defense against the herbivore is consistent. From the few studies available (Fontana *et al.*, 2009; Schausberger *et al.*, 2012; Pineda *et al.*, 2013) and this study, the pattern is that root colonization by beneficial microbes decrease the emission of volatile terpenoids following attack by caterpillars but increase the emission of volatile terpenoids following attack by cell-content feeders such as spider mites and phloem feeders such as aphids. More studies are, however, needed to confirm this pattern, and specially to elucidate what are the ecological consequences for the attraction of the different types of natural enemies, e.g. generalist versus specialist parasitoids. It is known that the parasitoid *M. mediator* is one of the most important natural enemies of the generalist herbivore *M. brassicae* (Lauro *et al.*, 2005), and is known to parasitize ca. 40 species of lepidopteran herbivores (Li *et al.*, 2006a; 2006b). Therefore, we conclude that rhizobacteria have a high potential to enhance the biocontrol of leaf-chewing herbivores based on an enhanced

attraction of parasitoids, but not on enhanced performance of parasitoids. This study shows the importance of a holistic approach by evaluating both plant direct and indirect defense to insect herbivores, to unravel the beneficial role of root-associated microbes in agricultural and natural ecosystems. Moreover, rhizobacteria have multiple effects on plants and their associated organisms, therefore measurement of both plant defense to insect herbivores and plant growth parameters in these multi-trophic interactions are crucial to determine if the benefits outweigh the costs.

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7

General discussion

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INTRODUCTION

In natural ecosystems, plants are under constant threat from pathogens and herbivorous insects that consume plant tissues. In parallel, plants interact with beneficial microbes colonizing the roots that can modify plant morphology and physiology, affecting plant interactions with detrimental organisms (Gange *et al.*, 2003; Bennett *et al.*, 2009; Pineda *et al.*, 2010). Root-associated microbes can induce physiological changes in the plant affecting its direct and indirect defense against herbivorous insects (Gange *et al.*, 2003; Guerrieri *et al.*, 2004; Van Oosten *et al.*, 2008; Hempel *et al.*, 2009; Valenzuela-Soto *et al.*, 2010; Hoffmann *et al.*, 2011; Pineda *et al.*, 2012; Schausberger *et al.*, 2012; Song *et al.*, 2013; Pangesti *et al.*, 2015). Studies of mycorrhizae-plant systems have shown general patterns in the effects of the microbes on plant direct defense to insect herbivores (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva *et al.*, 2009; Pineda *et al.*, 2010). However, molecular mechanisms with regards to plant signal transduction and gene expression underlying the effects on insect herbivores are difficult to explore in mycorrhizae-plant systems due to limitation of the available genetic tools of plants that can be colonized by the root-associated fungi. Although it is known that *Arabidopsis* and other Brassicaceae are not well colonized by mycorrhizal fungi, different groups of root-associated microbes are able to colonize *Arabidopsis* plants (Pieterse *et al.*, 1998; Ahn *et al.*, 2007; Lee *et al.*, 2012; Lundberg *et al.*, 2012; Van de Mortel *et al.*, 2012). Therefore this model plant can be used to explore the molecular mechanisms on how plants integrate responses when simultaneously interacting with beneficial root-associated microbes and insect herbivores.

Plants growth, development, reproduction and interactions with either beneficial or deleterious organisms are regulated by several plant hormones (Howe and Jander, 2008; Erb *et al.*, 2012; Pieterse *et al.*, 2012). The phytohormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) have been associated with regulation of plant-insect and plant-microbe interactions (Howe and Jander, 2008; Erb *et al.*, 2012; Pieterse *et al.*, 2012; Zamioudis and Pieterse, 2012), and therefore may also modulate microbe-plant-insect interactions (Van Oosten *et al.*, 2008). This thesis aimed to further our understanding of mechanisms regulating rhizobacterial-induced systemic resistance (ISR) to herbivorous insects by combining gene transcriptional, chemical, insect performance and behavioral approaches using the root-associated microbe-plant model system *Pseudomonas fluorescens* WCS417r (abbreviated as *Pf.* WCS417r) and *Arabidopsis thaliana* to study what changes are induced by rhizobacteria colonizing the roots in terms of gene transcription and secondary plant chemistry, and how this affects plant

defense to the leaf-chewing insects *Mamestra brassicae* and *Pieris brassicae*. Here, I discuss the most important findings of the experimental chapters and link them with relevant topics in the research of root-associated beneficial microbe-plant-insect interactions and address the following questions:

1. How does rhizobacterial colonization affect plant direct and indirect defense to leaf-chewing insects?

2. What is the role of plant hormones, in particular the jasmonic acid (JA) and ethylene (ET)- signaling pathways and crosstalk between these pathways in modulating gene transcriptional responses and the synthesis of defensive compounds in rhizobacteria-mediated induced systemic resistance (ISR) against leaf-chewing insects?

Plant-mediated interactions between root-associated microbes and insect herbivores with different feeding modes

Over the last decade, experimental evidence on mycorrhizae-plant systems has uncovered a general pattern in which the effects of the root-associated fungi on plant direct defense against insect herbivores are positive or neutral for phloem feeders and specialist chewing herbivores, but negative for generalist chewing herbivores (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva *et al.*, 2009), and raises the question whether plant colonization by different groups of root-associated microbes have similar effects on particular groups of insect herbivores. A set of studies using the rhizobacterium *P. fluorescens* WCS417r have shown that plant colonization by this rhizobacterium positively affects the generalist phloem feeders *Myzus persicae* (Pineda *et al.*, 2012) and *Bemisia tabaci* (Shavit *et al.*, 2013); no effect was recorded on the performance of the specialist phloem feeder *Brevicoryne brassicae* (Pineda *et al.*, 2012), the specialist leaf-chewing insects *Pieris rapae* (Van Oosten *et al.*, 2008) and *Pieris brassicae* (Pangesti *et al.*, 2015), and a negative effect was found on the generalist leaf-chewing insect herbivores *Spodoptera exigua* (Van Oosten *et al.*, 2008) and *Mamestra brassicae* (Pangesti *et al.*, 2015; Pangesti *et al.*, Chapter 4). Furthermore, colonization of *Arabidopsis* roots by another rhizobacterium, i.e. *P. fluorescens* strain SS101 (abbreviated as *Pf.* SS101), also results in a negative effect on the generalist leaf-chewing insects *S. exigua* and *M. brassicae* (Van de Mortel *et al.*, 2012; Pangesti *et al.*, Chapter 5). Taken together, the above studies show that colonization of plant roots by different groups of root-associated microbe i.e. mycorrhizae or rhizobacteria gives similar negative effects to the generalist leaf-chewing herbivores, no effect to the specialist leaf-chewing and

phloem-feeding herbivores and positive effects to the generalist phloem-feeding insect herbivores.

Root-associated microbes modify direct plant defense to insect herbivores with different feeding modes

- Plant hormones and induction of defense-associated genes

Intact responsiveness of plants to JA- and ET- signaling is important in the activation of ISR by mycorrhizae and most rhizobacteria, including the well-studied rhizobacterium *Pf.* WCS417r, against various pathogens (Pieterse *et al.*, 2002; Ahn *et al.*, 2007; Jung *et al.*, 2012; Weller *et al.*, 2012). The beneficial microbes mediate ISR by sensitization of plant systemic tissues via a mechanism called "priming", that allows plants to mount faster or stronger expression of defenses after subsequent pathogen attack (Van Wees *et al.*, 1999; Pieterse *et al.*, 2002; Verhagen *et al.*, 2004). It has been proposed by Van der Ent *et al.* (2009) that depending on the inducing agent and challenging organisms, priming of plant defense can be regulated via different mechanisms. Leaf-chewing insects and necrotrophic pathogens are generally more sensitive to JA- and ET-dependent defenses (De Vos *et al.*, 2005; Glazebrook, 2005), whereas piercing-sucking insects and biotrophic pathogens are generally more affected by SA-dependent defenses (Glazebrook, 2005; Mewis *et al.*, 2005; Pieterse and Dicke, 2007; Zarate *et al.*, 2007; Kusnierczyk *et al.*, 2008; Wu and Baldwin, 2010; Pieterse *et al.*, 2012). In studies of beneficial microbe-plant-insect interactions, the question is whether beneficial microbes prime plant defense via different phytohormone-regulated mechanisms upon attack by leaf-chewing or phloem-feeding insects.

Experimental evidence shows that colonization of *Arabidopsis* roots by the beneficial microbe *Pf.* WCS417r induces enhanced expression of the JA-regulated gene *LOX2*, and the JA/ET-regulated genes *PDF1.2* and *HEL* upon feeding by the generalist leaf-chewing caterpillars *S. exigua* or *M. brassicae*, and are associated with rhizobacteria-mediated ISR that negatively affects larval weight of these generalist leaf-chewing herbivores (Van Oosten *et al.*, 2008; Pangesti *et al.*, 2015) (Fig. 1). It is confirmed that in addition to its crucial role in rhizobacteria-mediated ISR against pathogens, intact JA and ET-signaling is also required for rhizobacteria-mediated ISR against the generalist leaf-chewing insect *M. brassicae*, as shown with mutant studies (Pangesti *et al.*, 2015; Pangesti *et al.*, Chapter 4). In line with the results of rhizobacteria-plant-insect interactions, root colonization of tomato by the mycorrhiza *Glomus mosseae* resulted in enhanced expression of the

anti-herbivore defense-related genes *lipoxygenase D* (*LOXD*), *allene oxide cyclase* (*AOC*) and *protease inhibitors* (*PI-I*, *PI-II*) upon attack by the leaf-chewer *Helicoverpa armigera* and may explain the negative effect on caterpillar weight (Song *et al.*, 2013). Induction of the genes was eliminated in the JA-biosynthesis mutant *spr2*, which indicates that intact JA is required for the mycorrhiza-mediated resistance to the herbivore. Whether ET-signaling also plays a role in mycorrhiza-mediated ISR against leaf-chewing insects still requires further study. It has been proposed in a study using *A. thaliana*, that simultaneous induction of JA- and ET signaling pathways makes the plant insensitive to subsequent suppression by SA signaling, with the JA/ET-regulated transcription factor ORA59 potentially mediating the process (Leon-Reyes *et al.*, 2010a). Although the results of Chapter 4 of this thesis suggest that a JA/ET-regulated transcription factor is crucial in modulating rhizobacteria-mediated ISR against caterpillars, ORA59 is not the only transcription factor responsible for the observed effect.

Interestingly, a study using *Arabidopsis* shows that despite the positive effect of rhizobacterium *Pf.* WCS417r colonization on a plant's response to the generalist phloem-feeding aphid *Myzus persicae*, the rhizobacteria-colonized plants are primed for stronger expression of the JA-regulated gene *LOX2* and the JA/ET-regulated gene *PDF1.2* following the herbivore attack (Pineda *et al.*, 2012). The authors propose that priming of the JA-regulated independent mechanisms most likely explains the positive effect of rhizobacteral colonization on the performance of *M. persicae*. Furthermore, herbivory by the phloem feeder *M. persicae* induced up-regulation of the SA-pathway marker gene *PR1*, and it is proposed that negative crosstalk between the SA- and JA-signaling pathways may inhibit the priming of the JA-regulated response on rhizobacteria-colonized plants. Supporting the proposed hypothesis, in *Arabidopsis* plants exogenous application of SA suppressed the expression of the JA/ET-regulated gene *PDF1.*2 (Leon-Reyes et al., 2010b). A study using tomato plants colonized by *Pf.* WCS417r also proposes the same mechanism explaining the increased performance of phloem-feeding *Bemisia tabaci* (Shavit *et al.*, 2013). Based on the above studies, in rhizobacteria-colonized plants, leaf-chewing caterpillars induced much higher changes in transcript levels of *LOX2* and *PDF1.2* compared to phloem-feeding aphids (Pineda *et al.*, 2012; Pangesti *et al.*, 2015), whereas phloem-feeding aphids induced a high expression of the SA-regulated gene *PR1* (Pineda *et al.*, 2012). Thus, in rhizobacteria-colonized plants, negative crosstalk between SA- and JA-signaling may affect the generalist phloem-feeding insects more than the leaf-chewing insects. Although rhizobacterial colonization also induced enhanced expression of JA- and ET-regulated genes upon feeding by phloem-feeding insects, the crucial role of both signaling pathways in plant-mediated interactions between rhizobacteria and

phloem-feeding insects still needs to be further investigated. It is known that the SA-signaling pathway inhibits JA-signaling by targeting transcription factor ORA59 (Van der Does *et al.*, 2013). It remains to be investigated if the JA/ET-regulated transcription factor ORA59 is also one of the targets through which phloem-feeding insects such as *M. persicae* inhibit priming of the JA-regulated response in rhizobacteria-colonized plants. The above studies indicate that due to the sophisticated strategy of phloem-feeding insects to suppress plant defense, performance of the phloem-feeding insects in rhizobacteria-colonized plants is likely to be more affected by increased nutritional quality induced by the rhizobacteria than by enhanced plant resistance induced by the rhizobacteria (Pineda *et al.*, 2010). Furthermore, a recent study shows that abscisic acid (ABA) signaling also plays a role in induced plant resistance to insect herbivores via positive crosstalk with JA signaling through the JA-regulated transcription factor MYC2 (Vos *et al.*, 2013). It is proposed that rhizobacterial colonization-mediated suppression of the *ABA* gene may explain the positive effect of the rhizobacterium on performance of the aphid *M. persicae* (Pineda *et al.*, 2012). Investigating crosstalk through JA/ET-targeted transcription factor(s) such as ORA59 with SA- and ABA- signaling pathways may provide new insight into the mechanism underlying differences in plant-mediated effects of root-associated microbes on leaf-chewing and phloem-feeding insects and also differential effects on generalist and specialist herbivores.

- Plant hormones and induction of plant defense compounds

In studies of microbe-plant-insect interactions, unraveling which plant signaling pathways are modulated by the presence of root-colonizing microbes and how this affects the synthesis of plant defensive compounds and plant response to insect herbivores is one of the major questions. Data in this thesis show that intact JA- and ET-signaling is required for the induction of ISR against leaf-chewing insects and that rhizobacterial colonization causes a shift to a stronger activation of the JA/ET-regulated ORA59-branch over the JA-regulated MYC2-branch (Pangesti *et al.*, Chapter 4). A previous study shows that the JA-regulated MYC2-branch is being prioritized over the ET-regulated ERF-branch upon herbivory (Verhage *et al.*, 2011). Activation of both JA- and ET-signaling pathways in rhizobacteria-colonized plants alone or in combination with herbivory also resulted in changes in glucosinolates composition (GLS) in comparison to non-colonized plants infested by the herbivore, in which the plants prioritized the JA-signaling pathway. It has been shown that treatment with methyl jasmonate (MeJA) increases the amount of indole GLS 3-4 fold (Brader *et al.*, 2001; Mikkelsen *et al.*, 2003), whereas the ET- and SA-signaling pathways only play a minor

role in this induction (Brader *et al.*, 2001). Interestingly, the experiments with mutants, presented in Chapter 4, show that activation of the JA signaling pathway induces the synthesis of aliphatic and indole GLS, whereas the ET pathway represses the synthesis of indole GLS. By modulating both the JA- and ET-pathways, *Pf.* WCS417r colonization alone and combined with caterpillar herbivory enhanced the synthesis induced aliphatic GLS and suppressed the synthesis of indole GLS (Fig. 1). I propose that the induced accumulation of aliphatic GLS and suppression of indole GLS synthesis could be one of the mechanisms underlying *Pf.* WCS417r-mediated ISR against the generalist leaf-chewing caterpillars, because several leaf-chewing caterpillars, including *M. brassicae,* are senstitive to aliphatic GLS (Beekwilder *et al.*, 2008; Kos *et al.*, 2012). In the case of rhizobacterium-plant-aphid interactions, the profile of defense compounds such as GLS may be differentially affected, as there is strong induction of SA-signaling upon feeding by aphids (Pineda *et al.*, 2012), that can suppress JA/ET-signaling involved in responses to rhizobacterial colonization of plant roots. Furthermore, phloem-feeding insects such as aphids can prevent ingestion of toxic plant compounds such as GLS-hydrolysis products, by minimising cell damage while feeding (Schoonhoven *et al.*, 2005). Therefore, phloem-feeding insects are less likely to be affected by the changes in GLS composition induced by rhizobacterial colonization compared to leaf-chewing insects.

Although most beneficial microbes mediate ISR via JA- and ET-dependent mechanisms, recent studies show that *Pf.* SS101 and *Bacillus* sp. L81 trigger ISR via SA-dependent mechanisms (Barriuso *et al.*, 2008; Van de Mortel *et al.*, 2012). Colonization of *A. thaliana* Col-0 roots by *Pf.* SS101 induced accumulation of both aliphatic and indole GLS in the shoots and in the roots (Van de Mortel *et al.*, 2012), and upregulation of camalexin synthesis in local and systemic tissues of rhizobacteria-colonized plants. Results of Chapter 4 and the study of Van de Mortel *et al.* (2012) indicate that different strains of a rhizobacterial species colonizing the roots could induce a unique mixture of plant defense compounds such as GLS and camalexin in systemic tissues that may contribute to different strengths of ISR to herbivorous insects from different feeding modes. Despite the difference in signal transduction underlying ISR via either JA/ET- or SA-dependent mechanisms, both require a functional regulatory protein NPR1 (Pieterse *et al.*, 1998; Ryu *et al.*, 2003; Stein *et al.*, 2008; Van de Mortel *et al.*, 2012). The protein NPR1 is also known to regulate crosstalk between SA- and JA-signaling (Spoel *et al.*, 2003). Furthermore, despite the difference in signal transduction underlying induction of ISR, individual or combined application of *Pf.* WCS417r and *Pf.* SS101 resulted in similar expression of the JA-regulated gene *MYC2*, the SA-regulated gene *WRKY70*

and the JA/SA-regulated gene *NPR1* in the roots (Pangesti *et al.*, Chapter 5). This may explain why individual and combined applications of both strains have similar effects on performance of the generalist caterpillar *M. brassicae.*

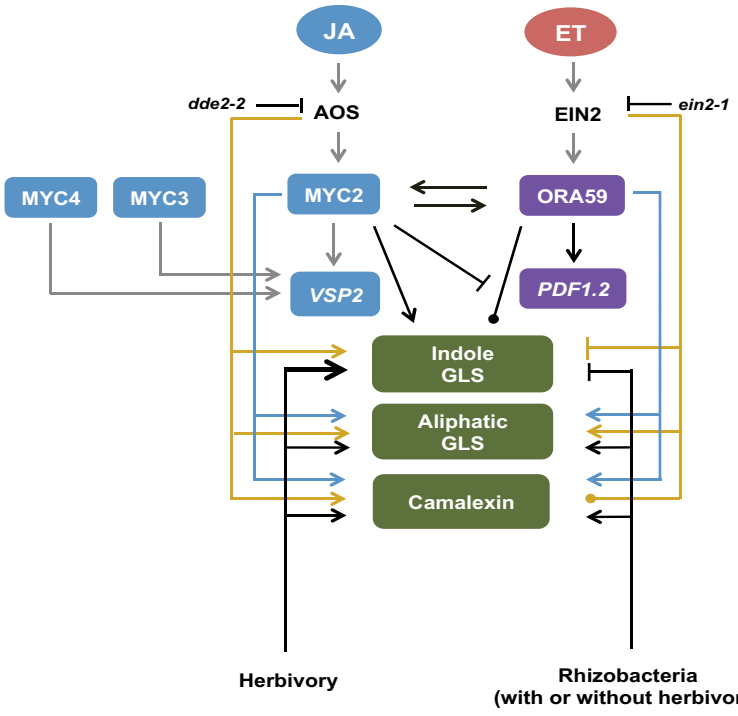

Figure 1. Working model of rhizobacteria-mediated induced systemic resistance (ISR) via jasmonic acid/ethylene (JA/ET)-dependent mechanisms based on studies in *Arabidopsis thaliana* Col-0. Rhizobacterial and herbivory induced modification of plant gene transcription, glucosinolates and camalexin biosynthesis represent in black lines. Central component of JA- and ET-signaling pathways regulatation of plant gene transcription and chemical biosynthesis represent in orange and blue lines. Grey lines represent results from the literature (Verhage *et al.*, 2011; Schweizer *et al.*, 2013). Arrows represent induction, truncated lines represent suppression; dotted line indicates no effect.

Root-associated microbes modify indirect plant defense against insect herbivores with different feeding modes

While studies on the effect of beneficial root-associated microbes on direct plant defense have revealed a general pattern depending on insect herbivore specialization and feeding mode, only limited information is available on how the microbes that colonize

plant roots affect third-trophic-level organisms, i.e. arthropod predators and parasitoids (Pineda *et al.*, 2010). Root-associated microbes modify plant physiology and, therefore, can have an impact on indirect plant defense against insects by modification of plant volatile emission. Few studies have shown that beneficial root-associated microbes can have positive or negative effects on the attraction of organisms from the third trophic level (Hoffmann *et al.*, 2011; Schausberger *et al.*, 2012; Pineda *et al.*, 2013). For instance, in bean plants, mycorrhizal colonization increases the emission of β-ocimene and β-caryophyllene and was associated with the attraction of a predatory mite (Schausberger *et al.*, 2012). In *Arabidopsis* plants, *Pf.* WCS417r colonization results in an increased attraction of the parasitoid *Microplitis mediator* to caterpillar-infested plants (Pangesti *et al.*, Chapter 6). Using the same plant species, *Pf.* WCS417r colonization modified HIPV emission after infestation by the generalist aphid *Myzus persicae* via JA-signaling and these changes reduced the attraction of the aphid parasitoid *Diaeretiella rapae* to the plants (Pineda *et al.*, 2013). Taken together, these two studies using *Pf.* WCS417r suggest that the effect of a certain root-associated beneficial microbe on indirect plant defense depends on the species of insect herbivores and their parasitoids.

Similar to the effects of root-associated microbes on arthropod predators and parasitoids, the effects of the microbes on emission of herbivore-induced plant volatiles (HIPV) also varies. Because the rhizobacterium *Pf.* WCS417r enhances the expression of JA-regulated genes following caterpillar herbivory (Van Oosten *et al.*, 2008; Pangesti *et al.*, 2015; Pangesti *et al.*, Chapter 4), I hypothesized that rhizobacterial treatment would also enhance the synthesis of HIPV, since plant volatile synthesis is also known to be regulated by JA-signaling (Dicke *et al.*, 2009; Dicke and Baldwin, 2010). In contrast, root colonization by *Pf.* WCS417r resulted in lower emission of the aromatics methyl salicylate, lilial and the terpene (*E*)-α-bergamotene upon feeding by the generalist caterpillar *M. brassicae*, in comparison to control plants infested with the caterpillars, although the total amount of volatile compounds in both treatments was similar (Pangesti *et al.*, Chapter 6). Showing the opposite pattern, plant colonization by the same root-associated bacteria increased the emission of HIPV upon feeding by the generalist aphid *M. persicae* (Pineda *et al.*, 2013). Based on results from several studies using different plant species (Fontana *et al.*, 2009; Schausberger *et al.*, 2012; Pineda *et al.*, 2013) the pattern is that beneficial root-associated microbes decrease the emission of volatile terpenoids following attack by caterpillars but increase the emission of volatile terpenoids following attack by cell-content feeders such as spider mites and phloem feeders such as aphids. Experimental evidence shows that feeding by the leaf-

chewing insects *Pieris rapae*, *Helicoverpa armigera*, *P. brassicae* and *M. brassicae* activate the expression of the JA-regulated transcription factor *MYC2* gene (Dombrecht *et al.*, 2007; Verhage *et al.*, 2011; Pangesti *et al.*, 2015). A recent study also indicates that the transcription factor MYC2 regulates the expression of terpene genes that may function in plant-insect interactions (Hong *et al.*, 2012). Furthermore, negative crosstalk occurs between the JA-regulated MYC2-branch and the JA/ET-regulated ERF-branch (Verhage *et al.*, 2011). It remains to be investigated whether activation of both JA- and ET-signaling pathways by rhizobacterium *Pf.* WCS417r colonization of plant roots is also responsible for modification of HIPVs via its crosstalk with the MYC2-branch.

Soil nutrients and rhizobacteria-mediated induced systemic resistance (ISR) against insect herbivores

Plants need mineral nutrients such as nitrogen, phosphate, and iron to grow and can actively obtain these compounds from soil. But plants also rely on root microbes to increase their access to nutrients as has been extensively shown for plant-mycorrhizae and plant-rhizobia interactions (Denison and Kiers, 2004; Breuillin *et al.*, 2010; Hoeksema *et al.*, 2010; Oldroyd *et al.*, 2011; Bonneau *et al.*, 2013). Therefore, the acquisition of, for instance, phosphate, nitrogen, or iron by plants and microbes may affect a wide range of plant-microbe interactions from mutualism to competition (Lemanceau *et al.*, 2009; Hoeksema *et al.*, 2010). In plant-mycorrhizae interactions, the availability of phosphate and nitrogen is known as one of the factors shaping mutualistic interactions (Breuillin *et al.*, 2010; Hoeksema *et al.*, 2010; Bonneau *et al.*, 2013), whereas in plant-rhizobia interactions, higher access to nitrogen is known to shape the mutualistic interactions (Denison and Kiers, 2004; Oldroyd *et al.*, 2011). In plant-rhizobacteria interactions, iron has been indicated to play a role in the interactions as one third of *Pf.* WCS417r-regulated genes are induced under iron limitation (Zamioudis, 2012). Iron is crucial for several plant metabolic processes, e.g. respiration and photosynthesis, and one of the most limiting nutrients for plant growth; however, the availability of iron in the soil is low (Lemanceau *et al.*, 2009; Palmer *et al.*, 2013).

Interestingly, under iron limiting conditions, plants increase the transcription rates of genes involved in iron homeostasis, in which MYB72 is one of the highest induced transcription factors (Palmer *et al.*, 2013). In line with this, in interactions between plants and root-associated microbes, activation of the root-specific transcription factor MYB72 is crucial in the early stage of ISR triggered by *Pf.* WCS417r and *Trichoderma*

asperellum T34 (Van der Ent *et al.*, 2008; Segarra *et al.*, 2009). The above facts indicate that there is a link between iron deficiency signaling and induction of ISR (Pieterse *et al.*, 2014). Results of this thesis show that rhizobacterium *Pf.* WCS417r-mediated enhanced expression of the JA/ET-regulated genes *PDF1.2* and *HEL* in plants grown under low-nutrient conditions, are associated with consistent negative effects of ISR on *M. brassicae* compared to high-nutrient conditions (Pangesti *et al.*, 2015). In line with this, ET signaling is known to regulate iron uptake (Lucena *et al.*, 2006) and is also involved in regulation of phosphate starvation in the plant (Lei *et al.*, 2011). Moreover, ET signaling is also crucial to establish interactions between plants and the endophytic fungus *Piriformospora indica* (Camehl et al., 2010), and to regulate nodulation in interactions between plant roots and rhizobia (Ma *et al.*, 2002). Taken together, this suggests that there is a link between ET-signaling and how plants intensify their interactions with beneficial microbes in nutrient-limiting environments, and through this intense interaction the root-associated microbes may trigger stronger ISR. In interactions between soybean and rhizobia, low nitrogen fertilization treatment resulted in higher root nodulation and stronger accumulation of the phytohormone JA, in comparison to high-fertilization treatment (Dean *et al.*, 2013). Results in this thesis show that in low nutrient conditions such as half-strength Murashige & Skoog (MS) media, *Pf.* WCS417r induced ISR consistently via JA/ET-mediated mechanisms that negatively affects the caterpillar *M. brassicae* (Pangesti *et al.*, Chapter 4). Activation of both JA- and ET-signaling enhanced the synthesis of aliphatic GLS and suppressed the synthesis of indole GLS, which may contribute to strong ISR against leaf-chewing insects, that are known to be sensitive to aliphatic GLS (Beekwilder *et al.*, 2008; Kos *et al.*, 2012). It remains to be investigated if nutrient deficiency signaling in the context of microbe-plant-insect interactions will also affect plant indirect defense against insect herbivores.

Studies of microbe-plant-insect interactions: future perspectives

Studies of microbe-plant interactions have revealed a fascinating role of root-associated microbes in the growth, development, and immune system of the host (Berendsen *et al.*, 2012; Chaparro *et al.*, 2012; Poupin *et al.*, 2013; Zamioudis *et al.*, 2013; Pieterse *et al.*, 2014). A rapidly growing body of evidence shows that root-associated microbes can modulate multiple physiological functions of the host, including defense against pathogens and acquisition of nutrients (Berendsen *et al.*, 2012; Turner *et al.*, 2013; Pieterse *et al.*, 2014). Studies in this thesis provide evidence that rhizobacteria

colonizing plant roots modify direct and indirect defenses of plants to leaf-chewing caterpillars. By modulating both the JA- and ET pathways, the rhizobacteria alter plant gene transcription and synthesis of secondary metabolites affecting insect herbivore performance. The fact that rhizobacteria- induced expression of JA/ET-regulated genes is more pronounced in nutrient-limiting conditions, which may explain the more consistent effect of rhizobacteria-mediated ISR on the generalist caterpillar *M. brassicae* is an interesting aspect that can be considered in the application of the beneficial root-associated rhizobacteria to increase plant immunity against leaf-chewing insects.

Apart from modification of plant secondary metabolism, plants also reorganize their primary metabolism following interactions with microbes (Weston *et al.*, 2012; Rojas *et al.*, 2014), and insect herbivores (Schwachtje and Baldwin, 2008; Gomez *et al.*, 2010). Results in Chapter 6 show that rhizobacterial colonization resulted in an increase in plant growth despite caterpillar feeding in comparison to control plants, and this effect of the rhizobacterium is only significant after longer caterpillar infestation. Furthermore, rhizobacterial colonization also results in an increased attraction of the parasitoid *Microplitis mediator* to caterpillar-infested plants. Taken together, the results suggest that rhizobacterial colonization can modify plant resource allocation to invest in both growth and indirect defense to increase fitness during caterpillar attack. Mechanistic studies in the field of microbe-plant-insect interactions mostly focus on how root-associated microbes modulate plant hormonal signaling and link this with expression of defense genes. It is known that in addition to its role in modulating plant defense, the plant hormone JA also regulates plant growth via its crosstalk with other plant hormones. Moreover, a recent study shows that application of methyl jasmonate (MeJA) to plant shoots triggers changes in the dynamics of carbon and nitrogen in the plant and increases the proportion of nitrogen allocation to roots (Gomez *et al.*, 2010). A future challenge is to investigate the role of hormonal pathways in regulating the re-organization of plant primary metabolism when plants are associated with beneficial root-associated microbes and insect herbivores and also link this with plant resistance to the herbivores that can yield fundamental understanding on how plants allocate resources when they interact with both beneficial and detrimental organisms.

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Summary

Plants as primary producers in terrestrial ecosystems are under constant threat from a multitude of attackers, which include insect herbivores. In addition to interactions with detrimental organisms, plants host a diversity of beneficial organisms, which include microbes in the rhizosphere. Furthermore, the interactions between plants and several groups of root-associated microbes such as mycorrhizae, plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) can affect plant interactions with foliar insect herbivores. The beneficial root-associated microbes are able to modify plant physiology by promoting plant growth and induced systemic resistance (ISR), in which the balance between both effects will determine the final impact on the insect herbivores. Using *Arabidopsis thaliana* Col-0, this thesis explores the molecular mechanisms on how plants integrate responses when simultaneously interacting with the rhizobacterium *Pseudomonas fluorescens* and the generalist and the specialist leaf-chewing insects *Mamestra brassicae* and *Pieris brassicae* respectively.

A literature review on the state-of-the-art in the field of microbe-plant-insect interactions (Chapter 2) explores how root-associated microbes and insect folivores can influence each other via a shared host plant. For more than a decade, both ecological and mechanistic studies mostly focused on exploring these belowground and aboveground interactions using single microbe and single herbivore species. The importance of increasing the complexity of the study system in order to understand the interactions in more natural situations is being emphasized. Furthermore, this review discusses the role of plant hormones in regulating plant growth and defense against folivores, while simultaneously being involved in associations with root-associated microbes.

Experimental evidence has shown patterns on the effects of mycorrhizal colonization on plant interactions with insect herbivores, and raises the question whether plant colonization by different groups of root-associated microbes has similar effects on particular categories of insect herbivores. In Chapter 3, plant-mediated effects of a non-pathogenic rhizobacterium on the performance of leaf-chewing insects, and the underlying mechanisms modulating the interactions, have been examined. Colonization of *A. thaliana* Col-0 roots by the bacterium *P. fluorescens* strain WCS417r resulted in decreased larval weight of the generalist leaf-chewing *M. brassicae*, and had no effect on larval weight of the specialist leaf-chewing *P. brassicae*. The crucial role of jasmonic acid (JA) in regulating rhizobacteria-mediated induced systemic resistance (ISR) against *M. brassicae* is confirmed by including plant mutants in the study. Interestingly, I also observed that rhizobacteria can induce systemic susceptibility to *M. brassicae* caterpillars. Comparison of *M. brassicae* performance and gene transcription in *A. thaliana* plants,

grown in potting soil or a mixture of potting soil and sand in a 1:1 ratio, shows that in a mixture of potting soil and sand, rhizobacterial treatment had a consistently negative effect on *M. brassicae*, whereas the effect is more variable in potting soil. Rhizobacterial treatment primed plants grown in potting soil and sand for stronger expression of JA- and ethylene-regulated genes *PDF1.2* and *HEL,* supporting stronger resistance to *M. brassicae*. Taken together, the results show that soil composition can be one of the factors modulating the outcome of microbe-plant-insect interactions.

Chapter 4 further addresses the mechanisms underlying rhizobacteria-mediated ISR against the generalist leaf-chewing *M. brassicae* by integrating plant gene transcription, chemistry and performance of *M. brassicae* in wild type *A. thaliana* Col-0 plants and mutants defective in the JA-pathway, i.e. *dde2-2* and *myc2*, in the ET pathway, i.e. *ein2-1*, and in the JA-/ET-pathway, i.e. *ora59*. Results of this study show that rhizobacterial colonization alone or in combination with herbivore infestation induced the expression of the defense-associated genes *ORA59* and *PDF1.2* at higher levels than activation by herbivore feeding alone, and the expression of both genes is suppressed in the knock-out mutant *ora59*. Interestingly, the colonization of plant roots by rhizobacteria alters the levels of plant defense compounds, i.e. camalexin and glucosinolates (GLS), by enhancing the synthesis of constitutive and induced levels of camalexin and aliphatic GLS while suppressing the induced levels of indole GLS. The changes are associated with modulation of the JA-/ET-signaling pathways as shown by investigating mutants. Furthermore, the herbivore performance results show that functional JA- and ET-signaling pathways are required for rhizobacteria-mediated ISR against leaf-chewing insects as observed in the knock-out mutants *dde2-2* and *ein2-1*. The results indicate that colonization of plant roots by rhizobacteria modulates plant-insect interactions by prioritizing the ORA59-branch over the MYC2-branch, although the transcription factor ORA59 is not the only one responsible for the observed effects of rhizobacteria-mediated ISR against leaf-chewing insects.

Taking a step further in increasing the complexity of the study system, Chapter 5 investigates how co-cultivation of *P. fluorescens* strains WCS417r and SS101 affects direct plant defense to the caterpillar *M. brassicae*. Inoculation of either *P. fluorescens* WCS417r or SS101 singly at root tips or simultaneously at two different positions along the roots resulted in a similar level of rhizobacterial colonization by each strain, whereas co-cultivation of both strains at either the root tips or at two different positions along the roots resulted in a higher colonization level of strain WCS417r compared to colonization by SS101. The results suggest that the site of inoculation influences

the direct interactions between the two strains in the rhizosphere, as also confirmed by *in vitro* antagonism assays in the absence of plants. Both upon single inoculation and co-cultivation of both strains at the same or different sites along the roots, the two rhizobacterial strains induced the same strength of ISR against the caterpillar *M. brassicae* and the same degree of plant growth promotion. In the roots, colonization by the two strains as single or mixed culture resulted in a similar gene expression pattern of up-regulation of *MYC2*, down-regulation of *WRKY70* and no effect on *NPR1* expression, genes representing JA-signaling, SA-signaling and the node of crosstalk between the two pathways, respectively. We hypothesize that both rhizobacterial strains use negative crosstalk between JA- and SA-pathways as mechanism to suppress plant immunity and establish colonization. This study shows that competitive interactions between rhizobacterial strains known to induce plant defense in systemic tissue via different signaling pathways, may interfere with synergistic effects on ISR and plant growth promotion.

While the effect of root-associated microbes on direct plant defense against insect herbivores has been studied previously, the effect of these microbes on indirect plant defense to herbivores is much less known. Chapter 6 explores how colonization by the rhizobacterium *P. fluorescens* strain WCS417r affects indirect plant defense against the generalist herbivore *M. brassicae* by combining behavioral, chemical and gene transcriptional approaches. The results show that rhizobacterial colonization of *A. thaliana* roots results in an increased attraction of the parasitoid *Microplitis mediator* to caterpillar-infested plants. Volatile analysis revealed that rhizobacterial colonization suppressed emission of the terpene (*E*)-α-bergamotene, and the aromatics methyl salicylate and lilial in response to caterpillar feeding. Rhizobacterial colonization decreased the caterpillar-induced transcription of the terpene synthase genes *TPS03 and TPS04*. Rhizobacteria enhanced both growth and indirect defense of plants under caterpillar attack. This study shows that rhizobacteria have a high potential to enhance the biocontrol of leaf-chewing herbivores based on enhanced attraction of parasitoids.

Taken together, the research presented in this thesis has shown how single or combined applications of rhizobacteria affect interactions of plants with leaf-chewing insects in terms of direct and indirect resistance. Furthermore, results presented in this thesis have revealed some of the molecular mechanisms underlying plant-mediated interactions between rhizobacteria and leaf-chewing insects that can be used in developing practical approaches by applying beneficial root-associated microbes for improving plant resistance.

Samenvatting

Planten zijn primaire producenten in terrestrische ecosystemen en staan onder constante dreiging van een verscheidenheid aan aanvallers, waaronder herbivore insecten. Naast interacties met schadelijke organismen zijn planten gastheer voor een verscheidenheid aan nuttige organismen, waaronder microben in de rhizosfeer. Bovendien kunnen interacties tussen planten en verschillende groepen wortel-bewonende microben zoals mycorrhiza, plantengroei-bevorderende rhizobacteriën (PGPR), en plantengroei-bevorderende schimmels (PGPF) de interacties tussen plant en bladvretende insecten beïnvloeden. De nuttige wortel-bewonende microben kunnen de fysiologie van planten wijzigen door plantengroei te bevorderen en systemische resistentie te induceren (ISR), waarbij de balans tussen beide effecten het uiteindelijke gevolg voor de herbivore insecten zal bepalen. Met behulp van *Arabidopsis thaliana* Col-0 worden in dit proefschrift de moleculaire mechanismen onderzocht met behulp waarvan planten reacties integreren wanneer ze gelijktijdig interacteren met de rhizobacterie *Pseudomonas fluorescens* en de generalistische bladvreter *Mamestra brassicae* (Kooluil) en de specialistische bladvreter *Pieris brassicae* (Groot Koolwitje).

Een literatuurstudie naar de meest recente ontwikkelingen op het gebied van microbe-plant-insect interacties (Hoofdstuk 2) onderzoekt hoe wortel-bewonende microben en blad-etende insecten elkaar kunnen beïnvloeden via een gedeelde waardplant. Gedurende ruim een decennium waren zowel ecologische als mechanistische studies vooral gericht op het onderzoeken van deze onder- en bovengrondse interacties aan één soort microbe en één soort herbivoor. Het belang van het vergroten van de complexiteit van het studiesysteem om deze interacties in een meer natuurlijke situatie te kunnen begrijpen wordt benadrukt in dit proefschrift. Bovendien komt in dit proefschrift de rol van plantenhormonen aan bod bij het reguleren van de plantengroei, de verdediging tegen bladvretende insecten en bij de interacties met wortel-bewonende microben.

Experimenteel bewijs heeft patronen aangetoond in de effecten van de kolonisatie van mycorrhiza op de interacties tussen planten en herbivore insecten, hetgeen de vraagt oproept of de kolonisatie van de plant door verschillende groepen wortel-bewonende microben vergelijkbare effecten zal hebben op bepaalde categorieën van herbivore insecten. In Hoofdstuk 3 zijn plant-gemedieerde effecten van een niet-pathogene rhizobacterie op de overleving en groei van bladvretende insecten, en de onderliggende mechanismen die de interacties moduleren, onderzocht. Kolonisatie van wortels van *A. thaliana* Col-0 door de bacterie *P. fluorescens* stam WCS417r resulteerde in een lager larvaal gewicht van de generalistische bladvreter *M. brassicae*, en had geen effect op het larvaal gewicht van de specialistische bladvreter *P. brassicae*. De cruciale rol van jasmijnzuur (JA) in

de regulatie van rhizobacterie-gemedieerde geïnduceerde systemische resistentie (ISR) tegen *M. brassicae* werd bevestigd door studies aan plantmutanten. Opmerkelijk is dat ik ook heb aangetoond dat rhizobacteriën systemische vatbaarheid kunnen induceren voor *M. brassicae* rupsen. Uit vergelijkingen van overleving en groei van *M. brassicae* en gentranscriptie in *A. thaliana* planten, gekweekt in potgrond of een mengsel van potgrond en zand in een 1: 1 verhouding, blijkt dat bij een mengsel van potgrond en zand de rhizobacteriële behandeling een consequent negatief effect had op *M. brassicae*, terwijl het effect meer variabel is in potgrond. Planten behandeld met rhizobacteriën en groeiend in een mengsel van potgrond en zand geven een sterkere expressie van de JA- en ethyleen-(ET)-gereguleerde genen *PDF1.2* en *HEL*, hetgeen de sterkere weerstand tegen *M. brassicae* rupsen ondersteunt. Samengevat laten de resultaten zien dat de bodemsamenstelling een factor is die de uitkomst van microbe-plant-insect interacties kan moduleren.

Hoofdstuk 4 gaat verder in op de mechanismen die ten grondslag liggen aan rhizobacterie-gemedieerde ISR tegen de generalistische bladvreter *M. brassicae* door de integratie van analyses van transcriptie van plantengenen, chemie en de overleving en groei van *M. brassicae* op wildtype *A. thaliana* Col-0 planten en mutanten met een defect in de JA-route, namelijk *dde2-2* en *myc2,* in de ET-route, namelijk *ein2-1,* en in de JA-/ET- route, namelijk *ora59*. De resultaten van deze studie tonen aan dat rhizobacteriële kolonisatie alleen, of in combinatie met aantasting door herbivoren, de expressie van de met verdediging geassocieerde genen *ORA59* en *PDF1.2* induceert tot een hoger niveau dan de activatie door bladvraat van een herbivoor alleen, en dat de expressie van beide genen is onderdrukt in de knock-out mutant *ora59*. Interessant is dat kolonisatie van de plantenwortels door rhizobacteriën de niveaus van de afweerstoffen van de plant, namelijk camalexin en glucosinolaten (GLS), verandert door het verhogen van de synthese van constitutieve en geïnduceerde niveaus van camalexin en alifatische GLS terwijl het de geïnduceerde niveaus van indool GLS onderdrukt. De veranderingen zijn geassocieerd met het moduleren van de JA-/ET- signaalroutes zoals aangetoond wordt door het onderzoek met mutanten. Daarenboven laten de resultaten van overleving en groei van de herbivoor zien dat functionele JA- en ET-signaalroutes vereist zijn voor rhizobacterië-gemedieerde ISR tegen bladvretende insecten zoals waargenomen in de knock-out mutanten *dde2-2* en *ein2-1*. De resultaten geven aan dat de kolonisatie van plantenwortels door rhizobacteriën de insect-plant interacties moduleert door de ORA59-tak te prioriteren over de MYC2-tak, hoewel de transcriptiefactor ORA59 niet de enige is die verantwoordelijk is voor de waargenomen effecten van rhizobacterie-gemedieerde ISR tegen bladvretende insecten.

In Hoofdstuk 5 wordt de volgende stap genomen in het verhogen van de complexiteit van het studiesysteem door te onderzoeken hoe co-cultivatie van *P. fluorescens* stammen WCS417r en SS101 de directe verdediging van de planten tegen de rups *M. brassicae* beïnvloedt. Inoculatie van hetzij *P. fluorescens* WCS417r of SS101 afzonderlijk op de wortelpunten of tegelijkertijd op twee verschillende posities op de wortels resulteerde in een vergelijkbaar niveau van rhizobacteriële kolonisatie door elke stam, terwijl co-cultivatie van beide stammen op ofwel de wortelpunten of op twee verschillende posities op de wortels resulteerde in een hoger kolonisatieniveau van stam WCS417r ten opzichte van de kolonisatie door SS101. De resultaten suggereren dat de plaats van inoculatie de directe interactie tussen de twee stammen in de rhizosfeer beïnvloedt, zoals ook bevestigd wordt door *in vitro* antagonisme-assays in afwezigheid van planten. Zowel bij enkelvoudige inoculatie en co-cultivatie van beide stammen op dezelfde of op verschillende plaatsen op de wortels, induceerden de twee rhizobacteriën dezelfde sterkte van ISR tegen de rups *M. brassicae* en bevorderden ze de plantengroei in dezelfde mate. In de wortels resulteerde kolonisatie door de twee stammen als enkele of gemengde kweek in een vergelijkbaar genexpressie patroon van up-regulatie van *MYC2*, down-regulatie van *WRKY70* en geen effect op *NPR1* expressie, genen die respectievelijk de JA-signalering, de Salicylzuur(SA)-signalering, en het knooppunt tussen de twee routes representeren. Onze hypothese is dat beide rhizobacteriestammen de negatieve interactie tussen de JA- en SA-routes als mechanisme gebruiken om de immuniteit van de plant te onderdrukken en kolonisatie tot stand te brengen. Deze studie toont aan dat competitieve interacties tussen rhizobacteriële stammen, waarvan bekend is dat ze de verdediging van planten in systemische weefsels via verschillende signaalwegen induceren, kunnen interfereren met synergetische effecten op de ISR en het bevorderen van de plantengroei.

Hoewel het effect van wortel-bewonende microben op de directe verdediging van planten tegen herbivore insecten eerder is onderzocht, is van het effect van deze microben op de indirecte verdediging van planten tegen herbivoren veel minder bekend. Hoofdstuk 6 onderzoekt hoe kolonisatie door de rhizobacterie *P. fluorescens* stam WCS417r de indirecte verdediging van planten tegen de generalistische herbivoor *M. brassicae* beïnvloedt door het combineren van gedrags-, chemische en gentranscriptiebenaderingen. De resultaten tonen aan dat rhizobacteriële kolonisatie van *A. thaliana* wortels resulteert in een verhoogde aantrekkingskracht van planten aangetast door rupsen voor de parasitaire sluipwesp *Microplitis mediator*. De analyse van vluchtige stoffen laat zien dat rhizobacteriële kolonisatie de emissie van het terpeen (*E*)-α-

bergamotene en de aromaten methyl salicylaat en lilial onderdrukt als reactie op vretende rupsen. Rhizobacteriële kolonisatie verminderde de rups-geïnduceerde transcriptie van de terpeen-synthese genen *TPS03* en *TPS04*. Rhizobacteriën verbeterden zowel de groei als de indirecte verdediging van planten die aangevreten werden door rupsen. Deze studie toont aan dat rhizobacteriën een hoog potentieel hebben om de biologische bestrijding van bladvretende herbivoren te verbeteren op basis van een verhoogde aantrekkingskracht voor parasitaire sluipwespen.

Samengevat toont het onderzoek gepresenteerd in deze thesis aan hoe enkelvoudige of gecombineerde toepassingen van rhizobacteriën de interacties van planten met bladvretende insecten beïnvloeden in termen van directe en indirecte weerstand. Bovendien onthullen de resultaten gepresenteerd in deze thesis een aantal van de moleculaire mechanismen die ten grondslag liggen aan plant-gemedieerde interacties tussen rhizobacteriën en bladvretende insecten die kunnen worden gebruikt bij het ontwikkelen van praktische benaderingen door het toepassen van gunstige wortel-bewonende microben die de weerstand van de plant verhogen.

Ringkasan

Tanaman sebagai produsen primer dalam ekosistem darat berada di bawah ancaman terus-menerus dari beragam organisme penyerang, salah satunya serangga herbivor. Selain interaksi dengan organisme yang merugikan, tanaman juga berinteraksi dengan beragam organisme yang menguntungkan, salah satunya mikroba yang hidup di akar. Interaksi antara tanaman dan organisme yang berasosiasi dengan akar, seperti: mikoriza, bakteri atau jamur yang dapat mempromosikan pertumbuhan tanaman (dikenal dengan istilah plant growth promoting bacteria/fungi (PGPR/PGPF)), dapat mempengaruhi interaksi tanaman dengan serangga herbivor melalui berbagai mekanisme. Mikroba menguntungkan yang hidup di akar dapat memodifikasi fisiologi tanaman dengan meningkatkan pertumbuhan tanaman dan juga menginduksi resistensi tanaman secara sistemik (dikenal dengan istilah Induced Systemic Resistance (ISR)), dan keseimbangan diantara kedua pengaruh tersebut menentukan status akhir dari interaksi tanaman dengan serangga herbivor. Menggunakan tanaman model *Arabidopsis thaliana* Col-0, tesis ini bertujuan mengeksplorasi mekanisme di level molekuler bagaimana tanaman mengintegrasikan responnya ketika secara bersamaan berinteraksi dengan bakteri yang berasosiasi dengan akar (rizobakteri) *Pseudomonas fluorescens* dan serangga herbivor generalis *Mamestra brassicae* dan spesialis *Pieris brassicae*.

Tinjauan literatur terbaru dalam bidang biointeraksi antara mikroba-tanaman-serangga (Bab 2) mengeksplorasi bagaimana mikroba akar dan serangga herbivor, yang secara fisik terpisah, dapat saling mempengaruhi melalui mekanisme yang dimediasi oleh tanaman inang. Selama lebih dari satu dekade, kajian-kajian ekologi dan mekanisme bidang biointeraksi antara mikroba-tanaman-serangga, sebagian besar berfokus pada eksplorasi menggunakan satu jenis mikroba dan satu jenis serangga herbivor. Pentingnya meningkatkan kompleksitas sistem kajian sebagai langkah untuk lebih memahami interaksi antar organisme di ekosistem alam menjadi fokus dari tinjauan literatur ini. Lebih jauh, peran hormon tanaman dalam mengatur pertumbuhan tanaman dan sistem pertahanan tanaman terhadap serangga ketika secara bersamaan juga berasosiasi dengan mikroba yang mengkolonisasi akar menjadi aspek yang didiskusikan.

Bukti penelitian menunjukkan pola mikoriza berpengaruh terhadap interaksi tanaman dengan serangga herbivore. Hal ini memunculkan pertanyaan apakah tanaman yang berasosiasi dengan mikroba akar dari kelompok lain memberikan pengaruh yang sama terhadap serangga herbivor dari kelompok tertentu. Bab 3 membahas tentang bakteri non-patogen yang mengkolonisasi akar tanaman model *A. thaliana* Col-0 terhadap performa serangga pengunyah daun, dan mekanisme yang mendasari interaksi antara mikroba-tanaman-serangga. Kolonisasi akar tanaman *A. thaliana* Col-

0 oleh rizobakteri *P. fluorescens* strain WCS417r berakibat pada menurunnya berat larva serangga generalis pengunyah daun *M. brassicae*, tetapi tidak memberikan efek pada berat larva serangga spesialis pengunyah daun *P. brassicae*. Peran penting hormon "jasmonic acid (JA)" dalam menginduksi resistensi tanaman secara sistemik (ISR) oleh rizobakteri yang efektif dalam menghambat performa *M. brassicae* telah dikonfirmasi dengan menggunakan tanaman mutan. Dalam penelitian ini ditemukan juga bahwa bakteri yang berasosiasi dengan akar juga dapat menyebabkan kerentanan sistemik pada tanaman (dikenal dengan istilah Induced Systemic Susceptibility (ISS)) melawan serangga pengunyah daun *M. brassicae*. Lebih lanjut, kajian performa *M. brassicae* dan ekspresi gen-gen tanaman *A. thaliana* Col-0 yang ditumbuhkan pada media tanah atau campuran media tanah dan pasir dengan perbandingan 1 : 1 menunjukkan bahwa kolonisasi rizobakteri pada tanaman yang tumbuh di media campuran tanah dan pasir, secara konsisten memberikan pengaruh negatif pada performa *M. brassicae*. Sedangkan pengaruh yang bervariasi ditemukan pada tanaman yang ditumbuhkan hanya dalam media tanah. Kolonisasi rizobakteri pada tanaman yang ditumbuhkan dalam media campuran tanah dan pasir, memicu ekspresi gen-gen *PDF1.2* dan *HEL* yang kuat. Ini mendukung fenomena performa negatif serangga herbivor *M. brassicae*. Ekspresi kedua gen tersebut diatur oleh hormone tanaman jasmonic acid (JA) dan ethylene (ET). Secara keseluruhan, hasil-hasil menunjukkan bahwa komposisi tanah dapat menjadi salah satu faktor yang mengatur biointeraksi rizobakteri-tanaman-serangga.

Bab 4 mengkaji lebih lanjut mekanisme yang mendasari ISR yang dimediasi oleh rizobakteri melawan serangga herbivor generalis *M. brassicae* dengan menggabungkan kajian pada level transkripsi gen-gen tanaman, kimia tanaman dan performa serangga herbivor *M. brassicae* yang mengkonsumsi tanaman *A. thaliana Col-0* dan tanaman mutan yang defektif di jalur metabolisme hormon JA: mutan *dde2-2* dan *myc2*, ethylene (ET): mutan *ein2-1*, JA dan ET: mutan *ora59*. Hasil penelitian menunjukkan kolonisasi bakteri akar sebagai perlakuan tunggal atau dikombinasi dengan infestasi serangga pengunyah daun *M. brassicae* dapat menginduksi ekspresi gen *ORA59* dan *PDF1.2* yang lebih tinggi dibandingkan perlakuan hanya dengan infestasi *M. brassicae* sebagai perlakuan tunggal, sementara itu ekpresi kedua gen terhambat pada tanaman "knock out" mutan *ora59*. Kedua gen tersebut diindikasikan berperan dalam sistem pertahanan tanaman terhadap organisme penyerang. Menariknya, kolonisasi rizobakteri memicu perubahan pada level metabolomik yakni sintesis senyawa yang penting untuk sistem pertahanan tanaman: camalexine dan glucosinolates (GLS), dengan meningkatkan sintesis senyawa camalexin dan aliphatic GLS pada level konstitutif dan pada saat yang

bersamaan menghambat sintesis senyawa indole GLS. Perubahan sintesis senyawa-senyawa tersebut berkaitan dengan modulasi jalur metabolisme hormon tanaman JA dan ET, seperti yang telah ditunjukkan pada kajian menggunakan tanaman mutan. Lebih jauh, kajian performa serangga herbivor menunjukkan bahwa jalur metabolisme JA dan ET yang fungsional diperlukan untuk terbentuknya ISR yang dipicu oleh kolonisasi rizobakteri. Hal ini diperkuat dengan performa serangga pemakan daun *M. brassicae* pada tanaman mutan *dde2-2* dan *ein2-1*. Hasil penelitian mengindikasikan kolonisasi akar tanaman oleh rizobakteri memodulasi interaksi tanaman dan serangga herbivor, dimana tanaman memprioritaskan jalur metabolisme JA cabang ORA59 dibandingkan cabang MYC2, walaupun ORA59 bukan merupakan satu-satunya faktor transkripsi yang berperan dalam menginduksi resistensi tanaman secara sistemik yang dipicu oleh kolonisasi rizobakteri dan efektif melawan serangga herbivor.

Dalam upaya melengkapi pemahaman kompleksitas mekanisme interaksi, Bab 5 mengkaji bagaimana rizobakteri *P. fluorescens* strain WCS417r dan SS101 yang bersamaan mengkolonisasi akar *A. thaliana* Col-0, mempengaruhi resistensi tanaman terhadap serangga pengunyah daun *M. brassicae.* Inokulasi rizobakteri *P. fluorescens* WCS417r atau SS101sebagai kultur tunggal pada ujung akar atau pada dua posisi berbeda di sepanjang akar menghasilkan pengaruh yang sama pada level kolonisasi dari masing-masing strain, sedangkan inokulasi kedua strain secara bersamaan pada ujung akar atau pada dua posisi yang berbeda di sepanjang akar menunjukkan level kolonisasi strain WCS417r lebih tinggi dibandingkan kolonisasi oleh strain SS101. Hasil tersebut mengindikasikan bahwa posisi dimana rizobakteri diinokulasi memiliki pengaruh pada interaksi langsung antara kedua strain di area rizosfer, sebagaimana dikonfirmasi oleh eksperimen uji antagonisme antara kedua strain secara *in vitro*, tanpa melibatkan tanaman. Inokulasi tunggal maupun inokulasi bersama kedua strain pada posisi yang sama maupun posisi yang berbeda sepanjang akar, kedua rizobakteri menginduksi ISR dengan kekuatan yang sama terhadap serangga herbivor *M. brassiae* dan juga memberikan pengaruh yang sama terhadap pertumbuhan tanaman. Kolonisasi kedua strain pada akar tanaman sebagai kultur tunggal ataupun campur menghasilkan pola ekspresi gen yang serupa: peningkatan ekspresi gen *MYC2*, penurunan ekpresi gen *WRKY70* dan tidak memberikan pengaruh pada ekspresi gen *NPR1*. Gen-gen tersebut secara berurutan mewakili JA-signaling, salicylic acid (SA)-signaling dan titik komunikasi ("crosstalk") antara kedua hormon signaling. Kami berhipotesis bahwa kedua strain rizobakteri menggunakan "negatif crosstalk" antara JA- dan SA-signaling sebagai mekanisme untuk menekan sistem kekebalan tanaman untuk bisa mengkolonisasi akar tanaman. Kajian

ini menunjukkan bahwa interaksi yang kompetitif antara strain rizobakteri yang secara individual dapat menginduksi sistem kekebalan tanaman melalui jalur metabolime hormon yang berbeda, dapat menghambat efek sinergisme ISR dan pertumbuhan tanaman.

Pengaruh rizobakteri terhadap sistem pertahanan tanaman secara langsung terhadap serangga herbivor telah banyak dikaji, sedangkah bagaimana pengaruh bakteri yang berasosiasi dengan akar tanaman tersebut terhadap sistem pertahanan tanaman secara tidak langsung melawan serangga herbivor, dengan melibatkan musuh alami dari serangga herbivor belum banyak diketahui. Bab 6 mengeksplorasi bagaimana rizobakteri *P. fluorescens* WCS417r yang mengkolonisasi akar tanaman berpengaruh terhadap pertahanan tanaman secara tidak langsung melawan serangga herbivor, dengan menggabungkan kajian perilaku musuh alami dari serangga herbivor, metabolit tanaman, dan transkripsi gen-gen tanaman. Hasil penelitian menunjukkan kolonisasi akar tanaman oleh rizobakteri berakibat pada meningkatnya ketertarikan serangga parasitoid *Microplitis mediator*, terhadap tanaman yang diserang oleh serangga pengunyah daun. Analisa senyawa volatil menunjukkan kolonisasi akar tanaman oleh rizobakteri menekan emisi senyawa terpene (*E*)-α-bergamotene, dan senyawa aromatics methyl salicylate dan lilial sebagai respon dari serangan pengunyah daun. Kolonisasi rizobakteri pada akar tanaman juga menekan ekspresi gen terpene synthase *TPS03* dan *TPS04* sebagai respon dari serangan serangga pengunyah daun. Rizobakteri juga meningkatkan pertumbuhan tanaman dan pertahanan tanaman secara tidak langsung ketika tanaman diserang oleh serangga herbivor. Kajian ini menunjukkan bahwa rizobakteri menjanjikan untuk diaplikasikan dalam meningkatkan biokontrol serangga herbivor pengunyah daun dengan meningkatkan daya tarik musuh alami dari serangga.

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Curriculum Vitae

Nurmi Puri Dwi Pangesti was born on February 2, 1978 in Bandung, Indonesia. She received her basic education in Yogyakarta and Jakarta, Indonesia. In 1996, she moved back to Bandung to study Biology at Institut Teknologi Bandung (ITB), and obtained the bachelor degree in 2001 with specialization in Microbiology. Her interest in research and possible application of microorganisms in various fields grew, and soon after her graduation, she worked as research assistant in the Laboratory of Applied Microbiology, Center of Life Sciences, ITB. She was involved in applied projects exploring beneficial soil microbes and their ability to control plant pathogens and promote plant growth, under supervision of Dr. I Nyoman P. Aryantha. Interest in agricultural research motivated her to pursue a Master degree and in 2004, following the successful application of a fellowship from the Netherlands Fellowship Programme (NFP), she joined the study programme Plant Biotechnology at Wageningen University, The Netherlands. Her interest in the field of plant-microbe interactions brought her to carry out a thesis in the Laboratory of Phytopathology (Wageningen University) on a project entitled "Molecular and biochemical basis of *Rhizoctonia solani* disease suppressive soils", under supervision of Dr. Marco Kruijt and Dr. Jos M. Raaijmakers. After she graduated in 2006, she continued working in applied research, and along the way she became interested in more fundamental research because this can provide valuable information to strengthen the application of technology. In 2010, she started PhD research in the Laboratory of Entomology (Wageningen University) on a project to study the effects of root-associated bacteria on plant defenses to insect herbivores and the underlying mechanisms, under supervision of Prof. dr. ir. Joop J.A. van Loon and Prof. dr. Marcel Dicke. The PhD project was funded by a fellowship from the Graduate School of Experimental Plant Sciences (EPS), Wageningen University. Results of the PhD studies exploring rhizobacteria-plant-insect interactions are presented in this book.

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Group: Laboratory of Entomology
University: Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Presenting plan at PhD lunch meeting	Dec 11, 2010
► **Writing or rewriting a project proposal**	
Writing a review or book chapter	
Writing a review article published in Frontiers of Plant Sciences, Oct. 2013, doi: 10.3389/fpls.2013.00414	Oct 23, 2012
► **MSc courses**	
Ecological Aspects of Biointeractions (ENT-30306)	2010
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD day Wageningen University	May 19, 2011
EPS PhD day University of Amsterdam	Nov 30, 2012
► **EPS theme symposia**	
EPS Theme Interaction between Plant and Biotic Agents, University of Amsterdam	Feb 03, 2011
EPS Theme Interaction between Plant and Biotic Agents, Wageningen University	Feb 10, 2012
EPS Theme Interaction between Plant and Biotic Agents, Utrecht University	Jan 24, 2013
► **NWO Lunteren days and other National Platforms**	
NAEM Lunteren	Feb 07-08, 2011
NWO Lunteren	Apr 04-05, 2011
Entomologendag	Dec 16, 2011
Entomologendag	Dec 14, 2012
Entomologendag	Dec 13, 2013
► **Seminars (series), workshops and symposia**	
Seminar WEES Evolution of Cooperation in Rhizosphere Mutualisms - E. Toby Kiers	Jun 17, 2010
Workshop Insect-Plant Interaction, Wageningen Universty	Nov 11, 2010
ExPectationS (EPS Career Day), Wageningen University	Nov 19, 2010
Seminar WEES Using insect systems to study how organisms interact with beneficial and harmful microbes - Nicole M. Gerardo	Apr 21, 2011
Workshop Insect-Plant Interaction, University of Amsterdam	Nov 23, 2011
Seminar WEES Viruses as beneficial symbionts of insects - Michael R. Strand	Apr 26, 2012
Workshop Insect-Plant Interactions, Wageningen University	Sep 24, 2013
► **Seminar plus**	
Georg Jander, Boyce Thompson Institute for Plant Research, USA	Jan 10, 2011
International symposia and congresses (highly recommended)	
14th International Symposium on Insect-Plant Interactions (SIP-14) 2011 , Wageningen, NL	Aug 13-17, 2011
Workshop European Science Foundation (ESF) "Plant-Microbe-Insect Interactions", Wageningen, NL	Aug 18-20, 2011
International workshop "Plant-Microbe-Insect Interactions", Spain	Sep 24-26, 2012
► **Presentations**	
Rhizosphere signalling course (oral)	Aug 23-25, 2010
14th International Symposium on Insect-Plant Interactions (SIP-14) (poster)	Feb 10, 2012
EPS Theme 2, Wageningen (oral)	Feb 10, 2012
Workshop ESF "Plant-Microbe-Insect Interactions, Spain (oral+poster)	Sep 24-26, 2012
Environmental Signaling course, Utrecht (poster)	Aug 26-28, 2013
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Nov 15, 2012
► **Excursions**	
Entomology PhD excursion	Oct 28-Nov 01, 2013
Subtotal Scientific Exposure	*16.4 credits**

CONTINUED ON NEXT PAGE

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Summer School 'Rhizosphere Signaling'	Aug 23-25, 2010
	Basic Statistics	Dec 14-16, 21, 22, 2010
	Autumn School 'Host-Microbe Interactomics'	Nov 01-03, 2011
	Summer School 'Environmental Signaling'	Aug 26-28, 2013
►	**Journal club**	
	Insect-Plant Interaction meeting	2010-2014
	PhD lunch meeting	2010-2014
	Priming meeting	2010-2014
►	**Individual research training**	
	Subtotal In-Depth Studies	*7.2 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Competence Assessment (CA)	Oct 25, 2011
	Techniques for Writing and Presenting Scientific Paper	Feb 14-17, 2012
	Adobe InDesign	Nov 06, 2013
	Voice and Presentation Skills	Feb 11 and 25, 2014
	Mobilising your Scientific Network	Mar 19 and 27, 2014
	Last stretch of the PhD	May 16, 2014
	Reviewing a Scientific Paper	Jun 10, 2014
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3,3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

Appendix A

Variation in plant-mediated interactions between rhizobacteria and caterpillars: potential role of soil composition

Nurmi Pangesti, Ana Pineda, Marcel Dicke & Joop J.A. van Loon

Laboratory of Entomology, P.O. Box 16, 6700 AA Wageningen, The Netherlands

Plant Biology 17: 474-483 (2015)

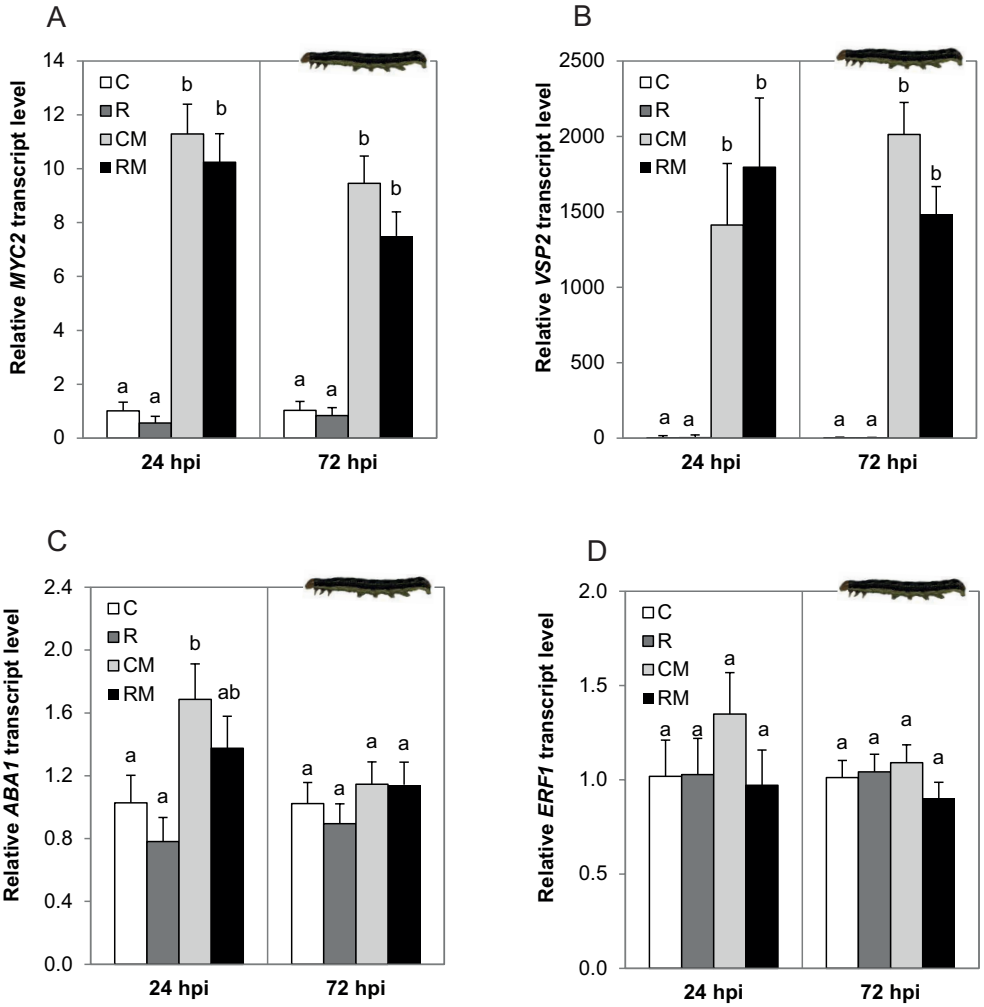

Figure S1. Relative transcript levels (mean ± SE) of several signal transduction pathway genes in local leaves of *A. thaliana* Col-0 control plants **(C)**, rhizobacteria-treated plants **(R)**, control plants infested with *M. brassicae* **(CM)**, or rhizobacteria-treated plants infested with *M. brassicae* **(RM)** at 24 and 72 hpi. Plants were grown in potting soil. Values were normalised relative to the reference genes *EF1α* and *FBOX*, and measured relative to the control plants (N = 5). Within each time point, different letters above bars indicate significant differences between treatments (Generalized Linear Model, $P < 0.05$, LSD pair-wise comparison).

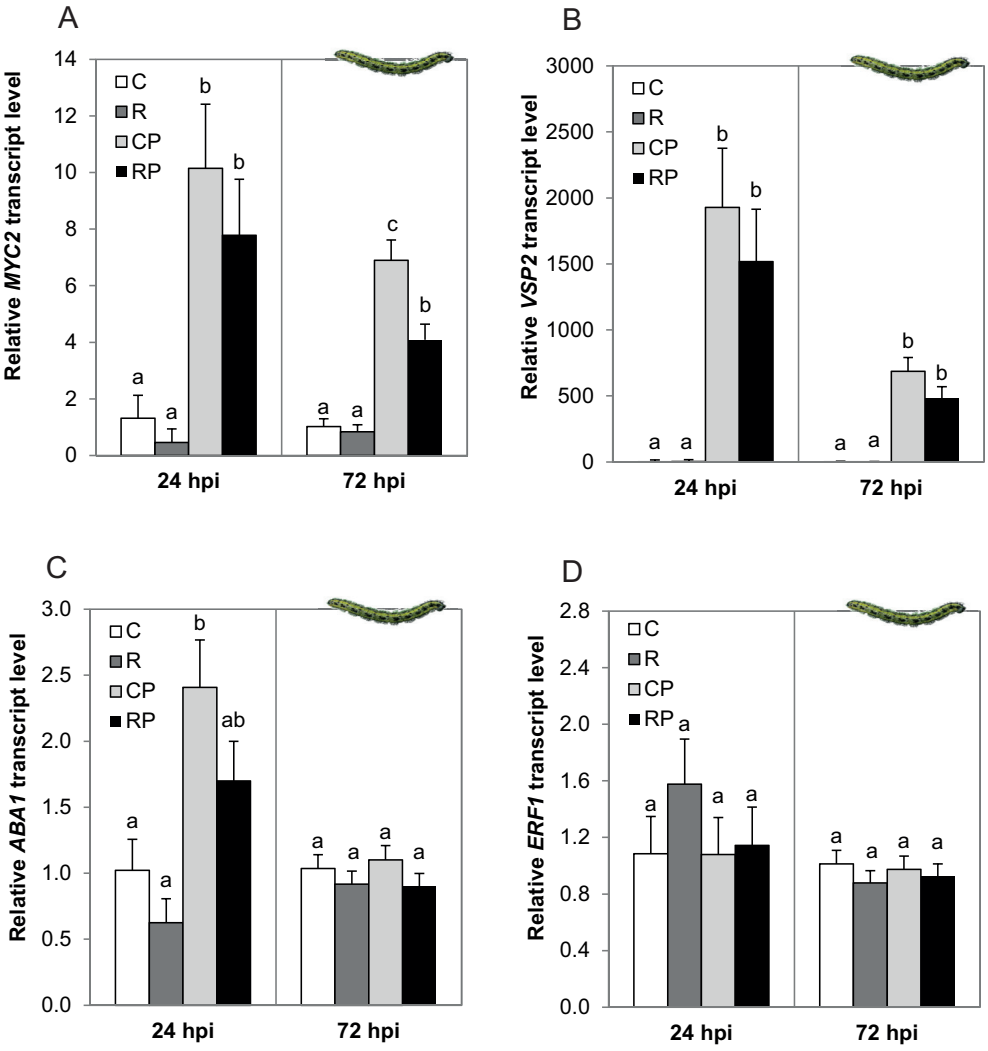

Figure S2. Relative transcript levels of genes in the JA signal transduction pathway in local leaves of *A. thaliana* Col-0 control plants **(C)**, rhizobacteria-treated plants **(R)**, control plants infested with *P. brassicae* **(CP)**, or rhizobacteria-treated plants infested with *P. brassicae* **(RP)** for 24 and 72 h. Plants were grown in potting soil. Transcript levels (mean ± SE) of tested genes which were normalised relative to reference genes of *EF1α* and *FBOX*, and measured relative to the control plants ($N = 5$). Within each time point, different letters above bars indicate significant differences between treatments (Generalized Linear Model, $P < 0.05$, LSD pair-wise comparison).

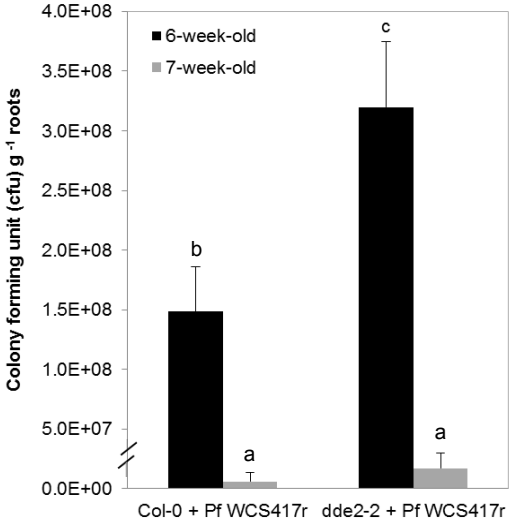

Figure S3. Colonisation of *P. fluorescens* WCS417r of the rhizosphere of *A. thaliana* Col-0 and *dde2-2* mutant on uninfested plants. The colonisation assays were performed on 6-week- and 7-week-old plants, which is equivalent to the age of plants use for insect performance assay of 6 dpi and 12 dpi. Data shown are means (± SE) of cfu.g^{-1} root fresh weight. different letters above bars indicate significant differences between treatments (Generalized Linear Model, $P < 0.05$, LSD pair-wise comparison).

Table S1. Primer sequences used in qRT-PCR

Gene ID	Gene		Sequence (5' --> 3')
At3g45140	***LOX2***	F	ACTTGCTCGTCCGGTAATTGG
		R	GTACGGCCTTGCCTGTGAATG
At5g44420	***PDF1.2***	F	CACCCTTATCTTCGCTGCTC
		R	GTTGCATGATCCATGTTTGG
At1g32640	***MYC2***	F	ATCCAAGTTCTTATTCGGGTC
		R	CGTCTTTGTCTCTCTGCTTCG
At5g24770	***VSP2***	F	TCAGTGACCGTTGGAAGTTGTG
		R	GTTCGAACCATTAGGCTTCAATATG
At5g67030	***ABA1***	F	TGGTCCTCTGTCTTTCTTGAC
		R	AGAGCATCGTCATCTTCAAAC
At1g27730	***ERF1***	F	CGAGAAGCTCGGGTGGTAGT
		R	GCCGTGCATCCTTTTCC
At3g04720	***HEL***	F	GAGAATAGTGGACCAATGCAG
		R	GTAGACCGATCGATATTGACCT
AT5G60390	***EF1***	F	TGAGCACGCTCTTCTTGCTTTCA
		R	GGTGGTGGCATCCATCTTGTTACA
AT5G15710	***FBOX***	F	TTTCGGCTGAGAGGTTCGAGT
		R	GATTCCAAGACGTAAAGCAGATCAA

Appendix B

Rhizobacteria-induced systemic resistance against a leaf-chewing herbivore is associated with induction of aliphatic glucosinolates and the ORA59-branch of the jasmonic acid signaling pathway

Nurmi Pangesti[1], Michael Reichelt[2], Judith E. van de Mortel[3], Eleni Kapsomenou[1], Joop J.A. van Loon[1], Marcel Dicke[1] & Ana Pineda[1]

[1]Laboratory of Entomology, [3]Laboratory of Phytopathology, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[2]Max Planck Institute for Chemical Ecology, Department of Biochemistry, 07745 Jena, Germany

Table S1. Contents of aliphatic and indole glucosinolates in µmol/g dry weight the shoot of *Arabidopsis thaliana* ecotype Col-0, JA-biosynthesis mutant *dde2-2* and ethylene-insensitive mutant *ein2-1*, in control plants **(C)**, rhizobacteria-treated plants **(R)**, control plants infested with *M. brassicae* **(CM)**, or rhizobacteria-treated plants infested with *M. brassicae* **(RM)**.

	Col 0				*dde2-2*				*ein2-1*			
	C	**R**	**CM**	**RM**	**C**	**R**	**CM**	**RM**	**C**	**R**	**CM**	**RM**
Aliphatic												
3MSOP	1.91 ± 0.11 **a**	2.54 ± 0.06 **b**	2.66 ± 0.17 **b**	3.27 ± 0.07 **c**	1.57 ± 0.11 **A**	2.05 ± 0.22 **b**	2.00 ± 0.06 **b**	1.51 ± 0.10 **a**	1.52 ± 0.11 **a**	1.79 ± 0.09 **ab**	2.01 ± 0.18 **b**	2.14 ± 0.09 **b**
4MSOB	15.80 ± 0.56 **a**	17.17 ± 0.51 **a**	20.94 ± 0.82 **b**	21.67 ± 0.98 **b**	12.61 ± 0.72 **a**	12.58 ± 0.10 **a**	10.86 ± 0.71 **a**	11.65 ± 0.57 **a**	12.78 ± 0.83 **a**	12.63 ± 0.62 **a**	15.20 ± 0.96 **b**	15.42 ± 2.00 **b**
7MSOH	0.54 ± 0.02 **a**	1.11 ± 0.05 **b**	0.56 ± 0.03 **a**	1.23 ± 0.06 **c**	0.66 ± 0.03 **b**	0.95 ± 0.02 **d**	0.55 ± 0.02 **a**	0.81 ± 0.02 **c**	0.53 ± 0.05 **a**	0.94 ± 0.06 **b**	0.62 ± 0.03 **a**	1.24 ± 0.07 **c**
4MTB	0.78 ± 0.08 **b**	0.63 ± 0.07 **b**	0.06 ± 0.02 **a**	0.17 ± 0.04 **a**	1.38 ± 0.15 **b**	1.16 ± 0.11 **b**	0.48 ± 0.12 **a**	0.67 ± 0.07 **a**	1.05 ± 0.17 **b**	1.03 ± 0.11 **b**	0.15 ± 0.02 **a**	0.11 ± 0.01 **a**
8MSOO	0.81 ± 0.05 **a**	3.38 ± 0.13 **b**	1.00 ± 0.07 **a**	3.88 ± 0.15 **c**	0.97 ± 0.08 **b**	2.52 ± 0.11 **d**	0.65 ± 0.06 **a**	2.02 ± 0.03 **c**	0.60 ± 0.05 **a**	1.98 ± 0.19 **b**	0.70 ± 0.11 **a**	2.78 ± 0.20 **c**
Sub total	19.84 ± 0.70 **a**	24.84 ± 0.60 **b**	25.23 ± 1.05 **b**	30.22 ± 0.95 **c**	17.20 ± 0.78 **b**	19.27 ± 0.28 **c**	14.54 ± 0.66 **a**	16.67 ± 0.64 **b**	16.48 ± 1.15 **a**	18.36 ± 0.82 **a**	18.68 ± 1.26 **ab**	21.70 ± 0.89 **b**
Indole												
4OHI3M	0.24 ± 0.01 **a**	0.30 ± 0.02 **b**	0.29 ± 0.01 **ab**	0.36 ± 0.03 **c**	0.16 ± 0.01 **a**	0.17 ± 0.01 **a**	0.16 ± 0.02 **a**	0.17 ± 0.00 **a**	0.18 ± 0.01 **a**	0.21 ± 0.01 **ab**	0.24 ± 0.01 **b**	0.32 ± 0.01 **c**
I3M	1.07 ± 0.08 **b**	0.66 ± 0.02 **a**	1.94 ± 0.11 **c**	1.11 ± 0.09 **b**	1.01 ± 0.08 **b**	0.57 ± 0.01 **a**	0.88 ± 0.08 **b**	0.52 ± 0.04 **a**	1.41 ± 0.13 **b**	0.84 ± 0.05 **a**	2.36 ± 0.11 **c**	2.09 ± 0.15 **c**
4MOI3M	0.25 ± 0.02 **a**	0.26 ± 0.02 **ab**	0.33 ± 0.00 **c**	0.31 ± 0.03 **bc**	0.27 ± 0.03 **a**	0.24 ± 0.03 **a**	0.29 ± 0.04 **a**	0.23 ± 0.02 **a**	0.26 ± 0.03 **b**	0.27 ± 0.01 **b**	0.12 ± 0.01 **a**	0.30 ± 0.03 **b**
1MOI3M	1.39 ± 0.07 **a**	1.45 ± 0.08 **a**	6.62 ± 0.20 **c**	3.79 ± 0.38 **b**	0.81 ± 0.08 **a**	0.87 ± 0.03 **ab**	1.13 ± 0.07 **c**	1.05 ± 0.08 **bc**	1.94 ± 0.28 **a**	1.57 ± 0.14 **a**	8.96 ± 0.62 **c**	7.43 ± 0.38 **b**
Sub total	2.95 ± 0.15 **a**	2.67 ± 0.11 **a**	9.19 ± 0.25 **c**	5.57 ± 0.50 **b**	2.25 ± 0.15 **bc**	1.86 ± 0.02 **a**	2.46 ± 0.16 **c**	1.97 ± 0.08 **ab**	3.79 ± 0.40 **a**	2.88 ± 0.13 **a**	11.67 ± 0.71 **c**	10.14 ± 0.51 **b**
Total	22.79 ± 0.71 **a**	27.51 ± 0.59 **b**	34.42 ± 1.20 **c**	35.79 ± 1.36 **c**	19.45 ± 0.82 **bc**	21.12 ± 0.29 **c**	17.00 ± 0.61 **a**	18.63 ± 0.64 **ab**	20.27 ± 1.52 **a**	21.24 ± 0.73 **a**	30.35 ± 1.87 **b**	31.83 ± 1.10 **b**

Aliphatic GLS: 3MSOP (glucoiberin), 4MSOB (glucoraphanin), 7MSOH (glucoibarin), 4MTB (glucoerucin), 8MSOO (glucohirsutin). **Indole GLS:** 4OHI3M (4-hydroxy-glucobrassicin), I3M (glucobrassicin), 4MOI3M (4-methoxy-glucobrassicin), 1MOI3M (neoglucobrassicin).

Table S2. Contents of aliphatic and indole glucosinolates content in µmol/g dry weight the shoot of *Arabidopsis thaliana* ecotype Col-0 and JA-regulated transcription factor mutant *myc2* and JA/ET-regulated transcription factor mutant *ora59* in control plants **(C)**, rhizobacteria-treated plants **(R)**, control plants infested with *M. brassicae* **(CM)**, or rhizobacteria-treated plants infested with *M. brassicae* **(RM)**.

	Col-0				*myc2*				*ora59*			
	C	**R**	**CM**	**RM**	**C**	**R**	**CM**	**RM**	**C**	**R**	**CM**	**RM**
Aliphatic												
3MSOP	1.89 ± 0.19 **a**	2.56 ± 0.19 **bc**	2.15 ± 0.17 **ab**	2.71 ± 0.09 **c**	1.55 ± 0.09 **a**	2.21 ± 0.10 **b**	1.76 ± 0.26 **ab**	2.72 ± 0.08 **c**	1.45 ± 0.20 **a**	2.32 ± 0.08 **bc**	1.95 ± 0.16 **b**	2.63 ± 0.12 **c**
4MSOB	12.84 ± 0.86 **a**	15.70 ± 1.26 **a**	18.89 ± 1.11 **b**	19.30 ± 0.51 **b**	9.92 ± 0.82 **a**	11.89 ± 0.78 **ab**	14.72 ± 1.75 **bc**	16.97 ± 0.59 **c**	9.14 ± 1.28 **a**	11.99 ± 0.58 **b**	15.83 ± 1.14 **c**	17.36 ± 0.49 **c**
5MSOP	0.95 ± 0.02 **a**	1.13 ± 0.10 **a**	1.42 ± 0.06 **b**	1.39 ± 0.04 **b**	0.81 ± 0.09 **a**	0.80 ± 0.04 **a**	1.08 ± 0.08 **b**	1.07 ± 0.05 **b**	0.69 ± 0.07 **a**	0.83 ± 0.04 **a**	1.24 ± 0.05 **b**	1.25 ± 0.04 **b**
7MSOH	0.29 ± 0.02 **a**	0.71 ± 0.07 **b**	0.37 ± 0.02 **a**	0.86 ± 0.02 **c**	0.26 ± 0.01 **a**	0.44 ± 0.03 **b**	0.30 ± 0.03 **a**	0.53 ± 0.01 **c**	0.27 ± 0.01 **a**	0.68 ± 0.08 **b**	0.41 ± 0.01 **a**	0.80 ± 0.05 **b**
4MTB	7.13 ± 0.98 **bc**	7.92 ± 0.76 **c**	3.72 ± 0.64 **a**	5.57 ± 0.28 **ab**	8.24 ± 0.63 **bc**	9.17 ± 0.32 **c**	3.69 ± 0.83 **a**	7.39 ± 0.15 **b**	7.69 ± 0.53 bc	8.87 ± 0.14 c	4.20 ± 0.59 a	6.30 ± 0.49 b
8MSOO	0.65 ± 0.05 **a**	2.49 ± 0.24 **b**	0.90 ± 0.07 **a**	3.13 ± 0.12 **c**	0.58 ± 0.03 **a**	1.57 ± 0.16 **b**	0.57 ± 0.09 **a**	1.75 ± 0.06 **b**	0.67 ± 0.05 **a**	2.38 ± 0.41 **b**	0.96 ± 0.04 **a**	2.52 ± 0.27 **b**
Sub total	23.76 ± 2.01 **a**	30.52 ± 2.41 **b**	27.46 ± 1.96 **ab**	32.97 ± 0.53 **b**	21.37 ± 0.82 **a**	26.09 ± 1.08 **ab**	22.11 ± 2.89 **a**	30.41 ± 0.70 **b**	19.91 ± 1.78 **a**	27.07 ± 0.82 **bc**	24.59 ± 1.75 **b**	30.85 ± 1.25 **c**
Indole												
4OHI3M	0.07 ± 0.01 **ab**	0.05 ± 0.01 **a**	0.14 ± 0.01 **c**	0.10 ± 0.03 **bc**	0.07 ± 0.01 **a**	0.09 ± 0.00 **a**	0.08 ± 0.01 **a**	0.09 ± 0.01 **a**	0.02 ± 0.01 **a**	0.03 ± 0.00 **a**	0.04 ± 0.00 **a**	0.04 ± 0.01 **a**
I3M	1.89 ± 0.15 **a**	1.84 ± 0.09 **a**	4.07 ± 0.31 **c**	3.12 ± 0.19 **b**	1.86 ± 0.02 **a**	1.48 ± 0.06 **a**	3.39 ± 0.65 **b**	2.89 ± 0.07 **b**	1.95 ± 0.04 **b**	1.51 ± 0.04 **a**	3.61 ± 0.23 **d**	2.81 ± 0.12 **c**
4MOI3M	1.19 ± 0.03 **a**	1.34 ± 0.06 **a**	1.12 ± 0.08 **a**	1.40 ± 0.12 **a**	1.19 ± 0.03 **a**	1.20 ± 0.02 **a**	1.20 ± 0.09 **a**	1.35 ± 0.02 **a**	0.89 ± 0.06 **a**	0.85 ± 0.02 **a**	0.83 ± 0.01 **a**	0.83 ± 0.09 **a**
1MOI3M	1.34 ± 0.24 **a**	1.76 ± 0.23 **a**	7.23 ± 0.81 **c**	4.86 ± 0.31 **b**	1.55 ± 0.04 **a**	2.07 ± 0.11 **a**	3.53 ± 0.77 **b**	3.58 ± 0.31 **b**	2.48 ± 0.25 **a**	2.21 ± 0.10 **a**	7.91 ± 0.88 **c**	6.10 ± 0.46 **b**
Sub total	4.49 ± 0.36 **a**	4.99 ± 0.35 **a**	12.56 ± 1.10 **c**	9.49 ± 0.55 **b**	4.67 ± 0.06 **a**	4.83 ± 0.12 **a**	8.20 ± 1.52 **b**	7.92 ± 0.38 **b**	5.34 ± 0.22 **a**	4.60 ± 0.08 **a**	12.38 ± 1.08 **c**	9.77 ± 0.63 **b**
TOTAL	28.25 ± 2.33 **a**	35.51 ± 2.33 **b**	40.02 ± 2.88 **bc**	42.46 ± 0.66 **c**	26.04 ± 0.85 **a**	30.92 ± 1.14 **a**	30.31 ± 4.37 **a**	38.33 ± 0.60 **b**	25.25 ± 1.99 **a**	31.67 ± 0.87 **b**	36.97 ± 1.85 **c**	40.62 ± 1.73 **c**

Aliphatic GLS: 3MSOP (glucoiberin), 4MSOB (glucoraphanin), 5MSOP (glucoalyssin), 7MSOH (glucoibarin), 4MTB (glucoerucin), 8MSOO (glucohirsutin). **Indole GLS:** 4OHI3M (4-hydroxy-glucobrassicin), I3M (glucobrassicin), 4MOI3M (4-methoxy-glucobrassicin), 1MOI3M (neoglucobrassicin).

Table S3. Variable Importance in the Projection (VIP) values of each glucosinolate compound in the shoot of diffferent *A. thaliana* lines. The VIP values relates to Projection to Latent Structures-Discrimination Analysis (PLS-DA)

Compound	Plant lines			Plant lines		
	Col-0	*dde2-2*	*ein2-1*	Col-0	*myc2*	*ora59*
Aliphatic						
3MSOP	0.918	**1.164**	0.850	0.960	0.789	**1.059**
4MSOB	0.917	0.812	0.686	0.931	0.802	0.940
5MSOP	**1.132**	**1.108**	**1.050**	0.943	0.923	0.996
7MSOH	0.937	**1.208**	0.855	**1.202**	0.980	**1.200**
4MTB	**1.178**	**1.098**	**1.103**	**1.026**	**1.316**	**1.063**
8MSOO	-	-	-	**1.236**	**1.066**	**1.154**
Indole						
4OHI3M	0.911	0.475	0.873	0.806	**1.068**	0.702
I3M	**1.064**	**1.135**	**1.123**	**1.024**	**1.026**	**1.107**
4MOI3M	0.740	0.780	**1.367**	0.720	0.993	0.571
1MOI3M	**1.121**	0.986	0.930	**1.039**	0.935	**1.029**

- VIP value > 1 written in bold

Table S4. Rhizobacterial colonization levels in roots of different plant lines

Experiment	Plant	Replicates	Colony forming unit (CFU) g^{-1} of roots
1	Col-0	2	$1.18*10^6$
	myc2	5	$4.85*10^5$
	ora59	4	$6.24*10^5$
2	Col-0	6	$2.65*10^5$
	dde2-2	6	$4.38*10^5$
	ein2-1	6	$3.56*10^5$
3	Col-0	5	$1.36*10^5$
	myc2	5	$1.14*10^5$
	ora59	5	$1.00*10^5$

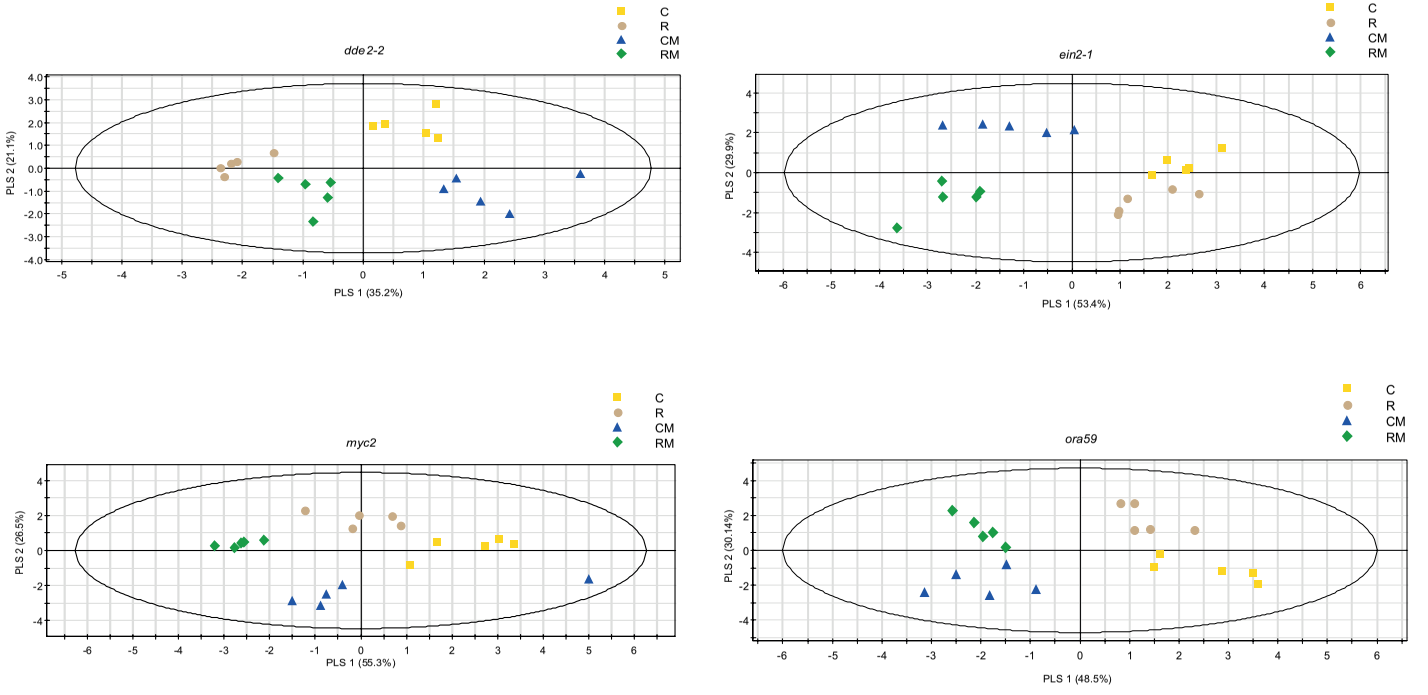

Figure S1. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparison of *Arabidopsis thaliana* Col-0 GLS profile from the shoot of mutants *dde2-2*, *ein2-1*, *myc2*, *ora59*. Treatments are control plants **(C)**, rhizobacteria-treated plants **(R)**, control plants infested with *M. brassicae* **(CM)**, or rhizobacteria-treated plants infested with *M. brassicae* **(RM).** Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point ($N = 5$ replicates) represents one sample from a pool of *A. thaliana* shoot collected from 5 plates.

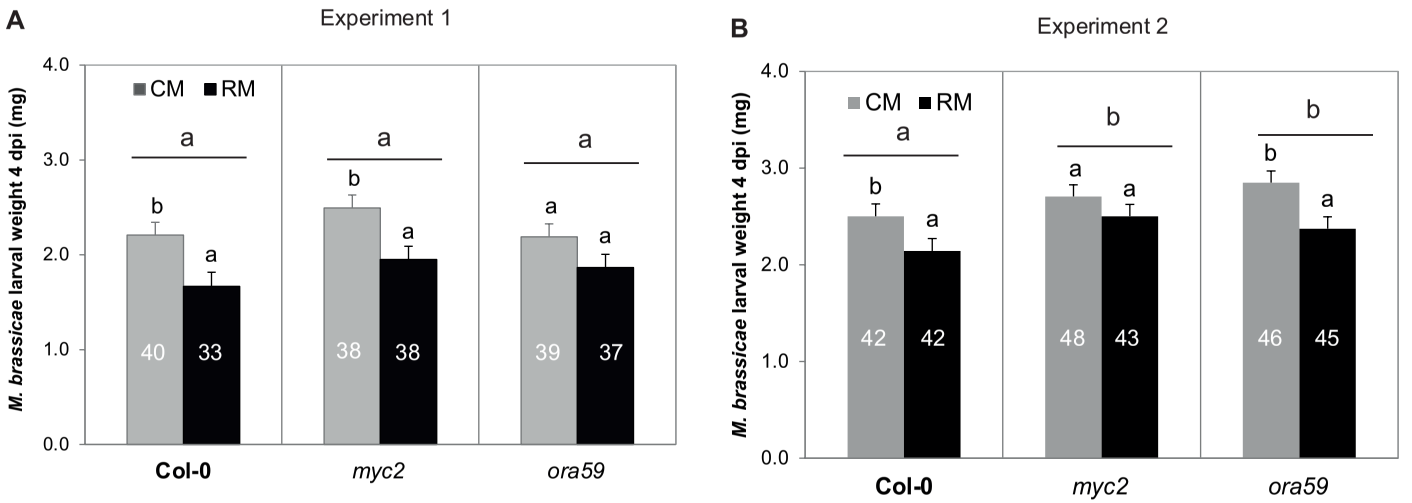

Figure S2. Performance of *M. brassicae* on control (CM) or rhizobacteria-treated plants (RM) in *A. thaliana* Col-0, mutants *myc2* and *ora59*. Larval weight was measured at 4 dpi, after infesting each plate with two neonate larvae. Numbers in each bar represent number of larvae suviving on the day of weight measurement. Data shown are means (± SE) of larval weight. Different letters over the bars indicate significant differences within a line, and letters over horizontal lines indicate differences between lines (LMM, $P < 0.05$, LSD test).

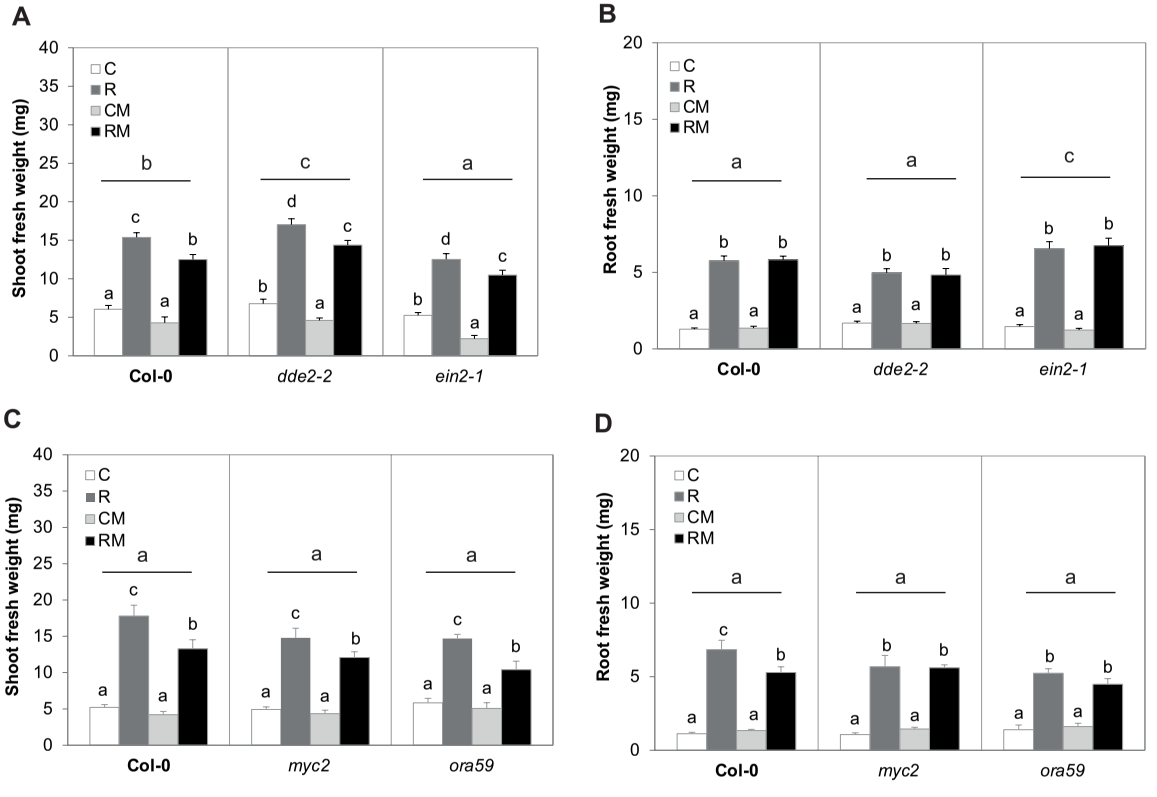

Figure S3. Shoot and root fresh weight (mean ± SE) of *Arabidopsis thaliana* Col-0, JA biosynthesis impaired mutant *dde2-2* and ethylene insensitive mutant *ein2-1*, **(A, B)** Col 0, *myc2*, *ora59* **(C, D)** of control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM) (N = 6 to 10 replicates). Comparisons are within line (one-way ANOVA, LSD post hoc test, $P < 0.05$), and between lines (two-way ANOVA. LSD post hoc test, $P < 0.05$).

Appendix C

Competitive interaction between beneficial *Pseudomonas fluorescens* strains in the rhizosphere does not affect the strength of plant growth promotion and induced systemic resistance against a leaf-chewing herbivore

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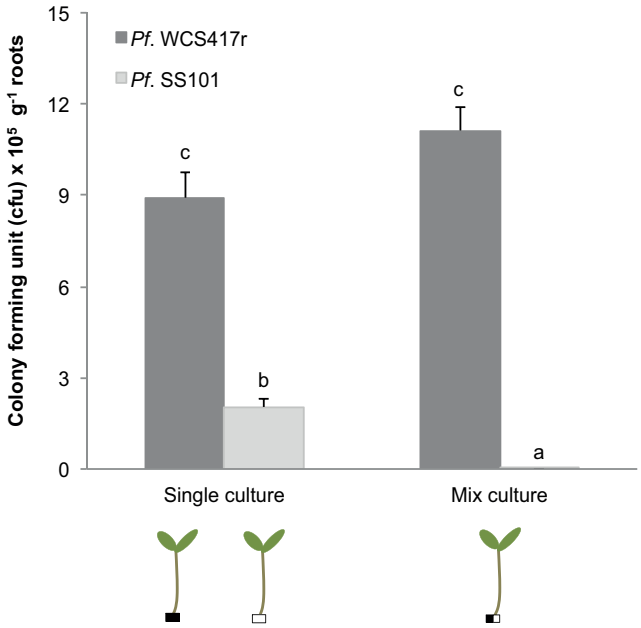

Figure S1. Abundance (mean ± SE) of *Pf.* WCS417r and *Pf.* SS101 in either single or combined inoculation of both strains stated in colony forming units (cfu) mg^{-1} of root fresh weight. Plant root tips were inoculated by 2 µl of 10 mM $MgSO_4$ for control, *Pf.* WCS417r, *Pf.* SS101 or mixture of both strains at a ratio of 1 : 1 (v/v) (N = 5 replicates). Different letters over the bars indicate significant differences between treatments (One-way ANOVA, $P < 0.05$, LSD test).

Appendix D

Rhizobacterial colonization of roots modulates plant volatile emission and enhances attraction of a parasitoid wasp to host-infested plants

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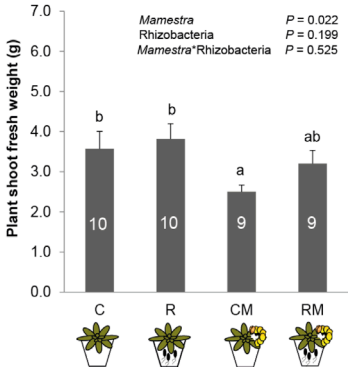

Figure S1. Shoot fresh weight of *A. thaliana* Col-0, for control plants (C), rhizobacteriatreated plants (R), control plants infested with *M. brassicae* (CM) and rhizobacteria-treated plants infested with *M. brassicae* (RM). The insect herbivore *M. brassicae* were feeding on the plants for 3 days.Data shown are means (± SE) of 4 pooled plant rosettes (two-way ANOVA, $P < 0.05$, LSD test; $N = 9$ -10). Different letters above bars indicate significant difference between treatments ($P < 0.05$).

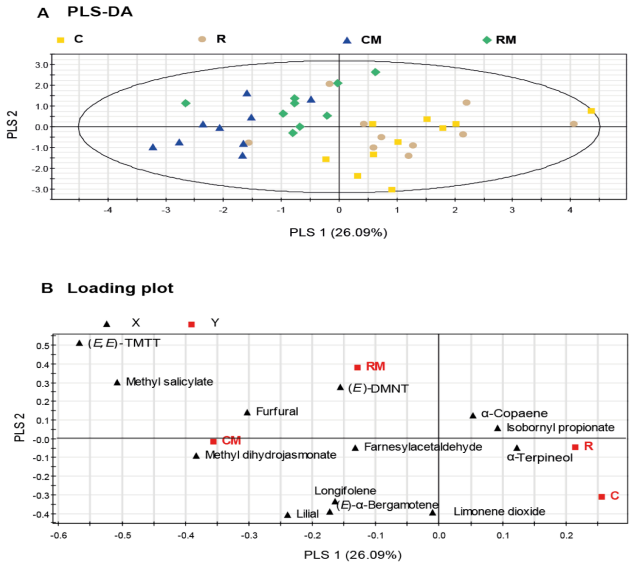

Figure S2. Projection to Latent Structures Discriminant Analysis (PLS-DA) comparing *A. thaliana* Col-0 volatile blends from control plants (C), rhizobacteria-treated plants (R), control plants infested with *M. brassicae* (CM), rhizobacteria-treated plants infested with *M. brassicae* (RM). (A) Grouping pattern of samples according to the first two principal components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample ($N = 9 – 10$ replicates); treatment with *M. brassicae* (CM/RM), three neonate larvae had been feeding for 3 days before volatile collection. (B) Loading plot of the first two components of PLS-DA, showing contribution of each volatile compound to the separation of the four treatments.

Table S1: Rhizobacterial colonization level in different plant batches used in this experiment

Treatments	Batch	*N* (plant)	Colony Forming Unit (CFU) g^{-1} of roots
Control plants	1	3	< 100
Rhizobacteria-treated plants	1	3	$1.7*10^5$
Control plants	2	2	< 100
Rhizobacteria-treated plants	2	2	$1.6*10^5$
Control plants	3	2	< 100
Rhizobacteria-treated plants	3	2	$1.6*10^5$
Control plants	4	2	< 100
Rhizobacteria-treated plants	4	2	$8.6*10^4$
Control plants	5	3	< 100
Rhizobacteria-treated plants	5	3	$5.9*10^5$

Table S2: Variable Importance in the Projection (VIP) values of each volatile organic compounds (VOCs) between treatments of control plants (C), rhizobacteria treated plants (R), control plants infested with *Mamestra brassicae* (CM) and rhizobacteria treated plants infested with *M. brassicae* (RM). The VIP values relates to Projection to Latent Structures-Discrimination Analysis (PLS-DA)

No	Compound	VIP		
		C-CM	R-RM	CM-RM
	Terpenoids			
1	(*E*)-DMNT	0.57	0.81	0.77
2	α-Terpineol	0.72	**1.02**	0.91
3	Limonene dioxide	0.78	0.89	0.77
4	Isobornyl propionate	0.54	0.96	0.78
5	α-Copaene	0.61	0.56	0.78
6	Longifolene	0.98	0.92	**1.17**
7	(*E*)-α-Bergamotene	0.85	0.43	**1.46**
8	(*E*,*E*)-TMTT	**1.70**	**2.07**	0.57
9	Farnesylacetaldehyde	0.54	0.22	0.48
	Aromatics			
10	Methyl salicylate	**1.78**	0.94	**1.51**
11	Lilial	0.75	0.85	**1.42**
	Others			
12	Furfural	0.98	0.88	0.23
13	Methyl *cis*-dihydrojasmonate	**1.16**	**1.25**	**1.16**

- VIP value > 1 written in bold.

Table S3: List of volatile organic compounds (VOCs) emitted by control plants (C), rhizobacteria treated plants (R), control plants infested with *Mamestra brassicae* (CM) and rhizobacteria-treated infested with *M. brassicae* (RM)

No	Compound	Volatile emissions are given as mean (± SE) of GC peak area divided by 10^5								Pairwise comparisons (t-test)							
		C		R		CM		RM		RM vs CM		C vs CM		R vs RM		R vs C	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	t	P	t	P	t	P	t	P
	Terpenoids																
1	(*E*)-DMNT	3.17	0.64	3.14	0.46	4.68	1.35	5.47	1.54	-0.36	0.72	-1.04	-1.04	-1.29	0.22	-0.89	0.38
2	α-Terpineol	1.25	0.18	1.00	0.18	1.36	0.19	1.03	0.09	-0.68	0.504	0.81	0.43	-0.64	0.53	0.97	0.34
3	Limonene dioxide	1.98	0.46	1.98	0.37	3.44	0.97	2.65	0.70	1.02	0.32	-1.49	0.16	0.76	0.46	-0.12	0.91
4	Isobornyl propionate	1.17	0.28	1.08	0.30	1.62	0.56	1.32	0.13	-0.93	0.37	0.72	0.48	-1.61	0.13	0.46	0.65
5	α-Copaene	0.50	0.06	0.49	0.10	0.46	0.08	0.49	0.08	-0.93	0.37	0.99	0.34	-0.88	0.39	0.94	0.36
6	Longifolene	2.32	0.25	2.28	0.29	2.78	0.29	2.21	0.17	1.69	0.11	-1.30	0.21	-0.11	0.91	0.21	0.84
7	(*E*)-α-Bergamotene	1.11	0.15	1.01	0.13	1.36	0.14	0.96	0.10	2.25	**0.04**	-1.34	0.20	-0.04	0.97	0.59	0.57
8	(*E*,*E*)-TMTT	8.09	1.40	8.86	2.01	41.09	9.32	38.97	12.89	0.40	0.69	-5.95	**<0.001**	-4.59	**<0.00**	-0.09	0.93
9	Farnesylacetaldehyde	163.62	61.95	129.63	44.47	255.54	93.21	158.90	46.77	0.56	0.58	-0.90	0.38	-0.35	0.73	-0.03	0.97
	Total terpenoids	183.21	61.70	149.47	44.11	312.32	91.66	212.01	45.26	.879	.392	-1.79	.092	-1.33	.200	.036	.972
	Aromatics																
10	Methyl salicylate	1.82	0.25	5.57	2.35	11.29	2.66	6.30	1.07	2.23	**0.04**	-7.62	**<0.001**	-1.63	0.12	-1.50	0.15
11	Lilial	5.96	1.65	3.61	0.59	8.73	2.04	4.14	0.63	2.18	**0.04**	-1.31	0.21	-0.86	0.40	0.99	0.34
	Total aromatics	7.78	1.67	9.18	2.34	20.02	2.62	10.44	1.61	3.64	**.002**	-4.18	**.001**	-1.14	.270	-.358	.725
	Others																
12	Furfural	11.67	1.15	13.33	1.75	21.23	4.43	18.05	2.61	0.31	0.76	-2.12	**0.05**	-1.50	0.15	-0.52	0.61
13	Methyl *cis*-dihydrojasmonate	9.86	1.64	8.29	2.17	18.34	2.26	13.21	1.91	1.58	0.13	-5.95	**0.02**	-2.28	**0.04**	0.66	0.52
	Total others	21.53	2.51	21.62	3.16	39.57	5.59	31.26	3.81	1.02	.323	-2.77	**0.013**	-1.99	0.063	.097	.924
	Total	212.52	61.68	180.27	46.03	371.91	94.78	253.71	45.84								

- $P < 0.05$ written in bold.

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