Plants under dual attack: consequences for plant chemistry and parasitoid behavior

Camille Ponzio

Plants under dual attack: consequences for plant chemistry and parasitoid behavior

Camille Ponzio

Thesis committee

Promotor
Prof. Dr Marcel Dicke
Professor of Entomology
Wageningen University

Co-promotor
Dr R. Gols
Researcher, Laboratory of Entomology
Wageningen University

Other members
Prof. Dr Bart P.H.J. Thomma, Wageningen University
Prof. Dr René A.A. van der Vlugt, Wageningen University
Dr Roxina Soler, Koppert Biological Systems, Berkel en Rodenrijs
Dr Ian A. Kaplan, Purdue University, United States of America

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

Plants under dual attack: consequences for plant chemistry and parasitoid behavior

Camille Ponzio

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 21 June 2016
at 11 a.m. in the Aula.

Camille Ponzio
Plants under dual attack: consequences for plant chemistry and parasitoid behavior
192 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

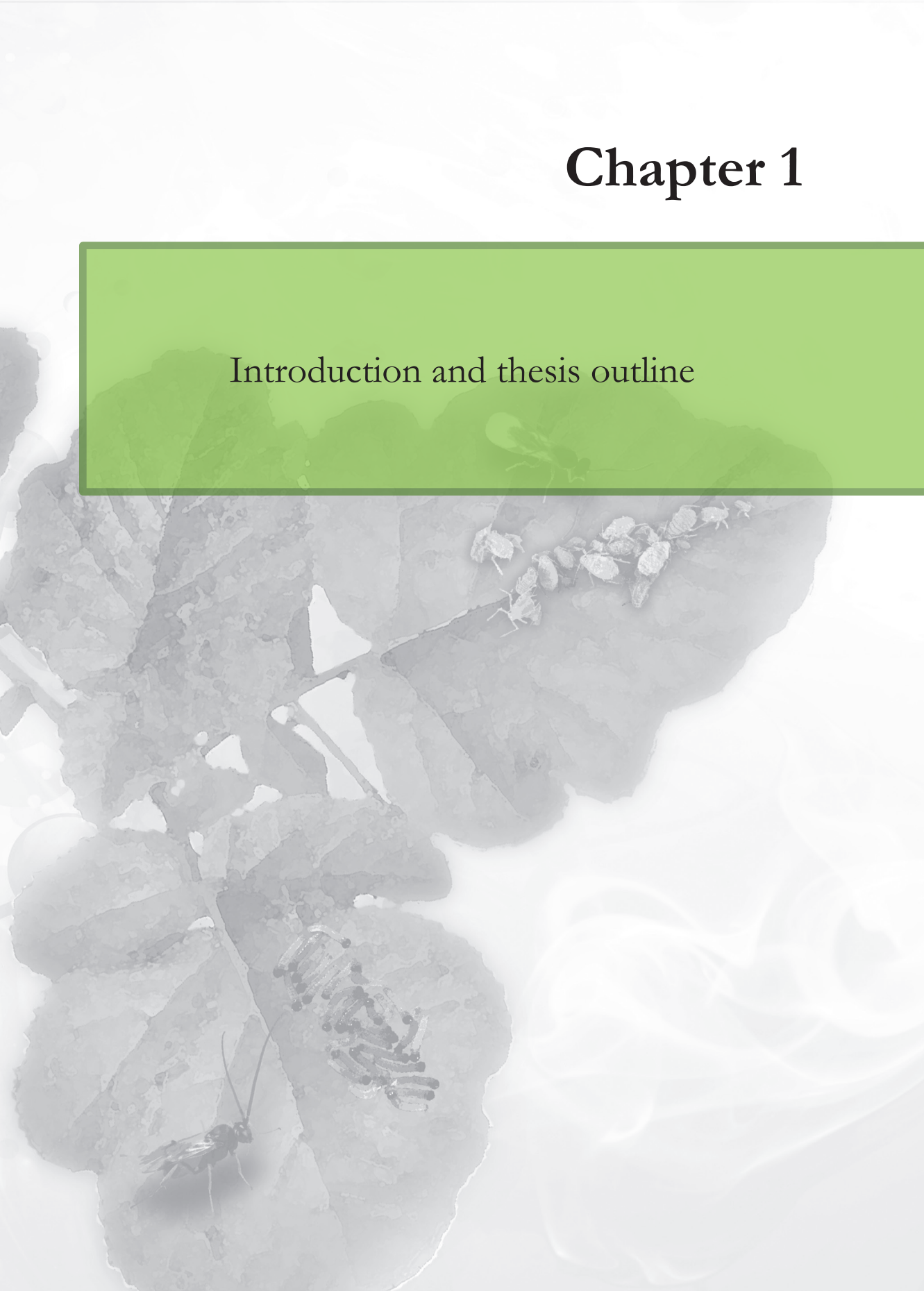
ISBN 978-94-6257-771-8, DOI 10.18174/377707

To my family

Contents

Chapter 1

Introduction and thesis outline



Introduction

Plants are constantly releasing a wide spectrum of volatile organic compounds (VOCs) into the atmosphere, which result from numerous physiological processes, and which play key roles in mediating interactions between plants and their associated community members. Over 1000 of these low molecular weight volatile compounds are known (Dudareva *et al.*, 2004), with a portion being constitutively emitted by healthy plants, while others are released or synthesized 'de novo' only after damage or stress (Pare & Tumlinson, 1999). These volatiles play a role in reproduction by attracting pollinators or seed dispersers (Pichersky & Gershenzon, 2002) and have a role in defense against many aggressors, especially when induced in response to stress. Plant VOC emissions can be modified substantially in response to stresses imposed by their environment and organisms within that environment. These induced volatile chemical products can confer protection against abiotic stress (Loreto & Schnitzler, 2010), have repellent effects on herbivorous insects (Unsicker *et al.*, 2009) and a microbicidal effect on plant pathogens (Croft *et al.*, 1993), can be exploited as cues by herbivores to locate suitable host plants for feeding and reproduction (Bruce *et al.*, 2005), and herbivore-induced plant volatiles can be used by natural enemies of the herbivores, such as parasitoid wasps or predators, as foraging cues to locate host herbivore-infested plants (Vet & Dicke, 1992; Dicke, 1999).

However, natural situations are infinitely more complex than what is often studied in lab-based systems. Multiple herbivores can co-occur on the same plant, and can be separated spatially or temporally, while plant pathogens represent another class of ubiquitous attackers. There has been increasing focus on the effects of multiple stress on the performance and preference of associated insect community members, notably in the Brassicaceae (Stam *et al.*, 2014), yet induced VOC emissions have been extensively studied primarily in response to individual abiotic or biotic stress, with little known of the mechanisms of VOC induction during dual stress. However, as the effects of multiple stress are expressed at the molecular level, it is expected that plant defense responses can be affected at other levels as well, such as induced volatile production, and lead to changes in plant interactions with their community members (Dicke *et al.*, 2009).

Plant interactions with attacking herbivores and pathogens are regulated by a complex regulatory network mediated by phytohormones. While many phytohormones are involved, it is well established that the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways play key roles in modulating plant defense responses, affecting gene transcription and biosynthesis of volatile and non-volatile metabolites (Howe &

Jander, 2008; Grant & Jones, 2009; Pieterse *et al.*, 2012). However, there is crosstalk between these signaling pathways, with simultaneous or sequential induction of multiple pathways leading to antagonistic or synergistic interactions (Pieterse *et al.*, 2012). This regulatory mechanism allows plants to fine tune their defense response to pathogen or herbivore attack (Koornneef & Pieterse, 2008; Verhage *et al.*, 2010). Crosstalk has been especially well documented for the SA and JA defense signaling pathways, which are often mutually antagonistic, with induction of the SA signaling pathway having particularly strong inhibitory effects on subsequent JA signaling pathway induction (Stout *et al.*, 2006; Koornneef & Pieterse, 2008).

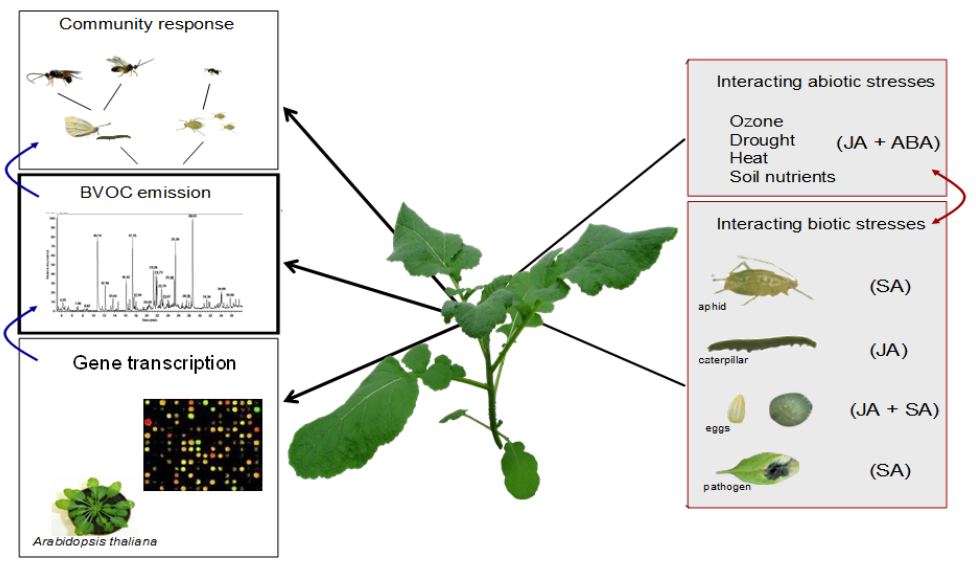


Figure 1. The A-BIO-VOC project's multidisciplinary approach to study the ecology of plant volatiles in response to biotic and abiotic stress, with this thesis having an emphasis on the impact of dual biotic-attack situations.

It is in this context of crosstalk that this thesis was carried out, as part of the project "Induction of plant VOC emission by biotic and abiotic stresses and consequences for community ecology: a multidisciplinary approach" (A-BIO-VOC), part of the collaborative European research program EuroVOL. This EU-wide project aimed to unravel the effects of dual attack by biotic and abiotic stresses on the induction and production of VOCs, and took a multidisciplinary approach to the ecology of plant volatiles and this at several levels of biological integration. This was achieved by investigating the interactions of biotic and/or abiotic challengers on gene expression, plant chemistry (metabolomics and plant volatiles) as well as studying the effects on

higher trophic levels (Fig. 1). As a contribution to the A-BIO-VOC project, the aim of this thesis was to unravel the effects of interacting biotic stresses – both insect herbivores and plant pathogens – on induced plant volatile blends, and their subsequent effects on parasitoid foraging. Moreover, this approach was completed with an analysis of the plant metabolome after one specific dual attack scenario.

Research Objectives

The main objective of this thesis was to investigate how biotic stresses interact to affect plant chemistry, particularly in the process of plant volatile induction and emission, and the effects these interactions have on the third trophic level. To achieve this, I focused on several aspects. A first aim was to determine if dual infestation with attackers that theoretically induce opposing defense signaling pathways had an impact on volatile-mediated foraging, by investigating the impact of various secondary, non-host attackers. As herbivore density appeared as an important factor, I then investigated the impact of herbivore density on plant metabolism (volatile and non-volatile) and on different members of the third trophic level. Finally, I explored if plant pathogen challenge exerts the same force as non-host herbivores in modifying tritrophic interactions.

Study system

I used a naturally occurring study system consisting of the brassicaceous plant *Brassica nigra* and some of its associated community members (Fig. 2), which are described in detail below. The biotic stressors used in this thesis were selected on the basis of the defense signaling pathways they are commonly thought to induce. It is against the crosstalk background described previously that the research contained in this thesis was carried out; while *Pieris brassicae* caterpillar feeding is primarily activating JA-mediated defenses, all the secondary attackers (*P. brassicae* eggs, *Brevicoryne brassicae* aphids, *Xanthomonas campestris* bacterial pathogens) were chosen because they induce the SA signaling pathway (Ton *et al.*, 2002; Kusnierczyk *et al.*, 2008; Bruessow *et al.*, 2010), though to varying degrees. In the context of dual attack, prioritization of one pathway over another, via crosstalk, would be expected to have a strong effect on subsequent plant responses.

Plant

Black mustard, or *Brassica nigra*, is an annual weedy brassicaceous plant, which occurs

naturally in the Netherlands, where it usually is present from May to October, in large stands. As a member of the Brassicaceae, *B. nigra* contains high levels of glucosinolates, which are important defensive secondary compounds conferring protection against many generalist herbivores (Hopkins *et al.*, 2009). It is therefore primarily attacked by specialist herbivores, which have developed adaptations to excrete or even sequester glucosinolates (Rask *et al.*, 2000; Wittstock *et al.*, 2004; Winde & Wittstock, 2011). Breakdown products of glucosinolates such as isothiocyanates and nitriles are also found in the volatile blends emitted by damaged plants (Geervliet *et al.*, 1997; Gols *et al.*, 2009) and can play a role in the attraction of parasitoids of herbivores specialized on brassicaceous plants (Bradburne & Mithen, 2000; Mumm *et al.*, 2008).

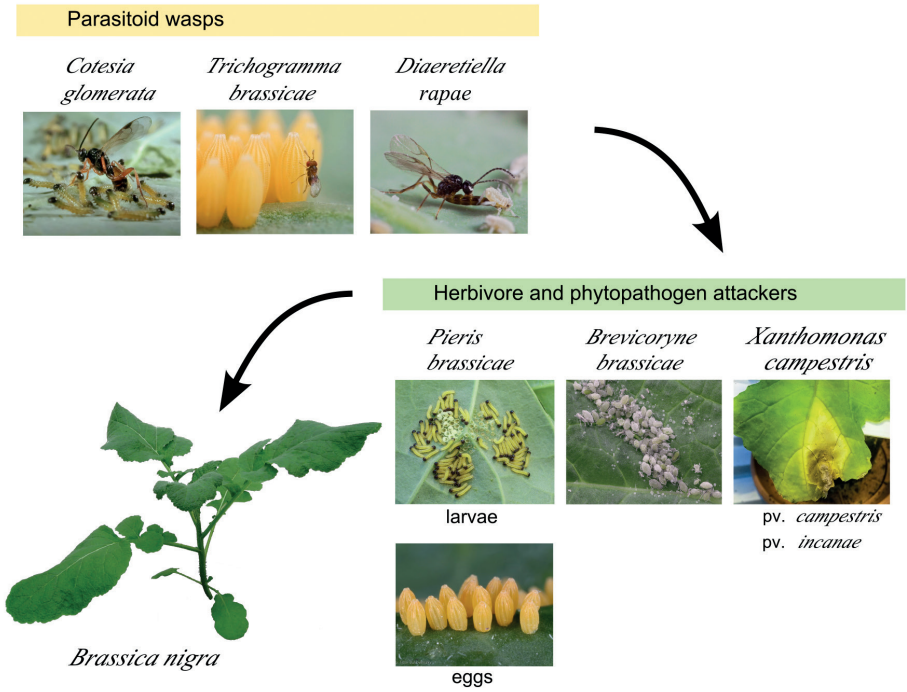


Figure 2. The tritrophic system used in this thesis. Brassica nigra plants in the vegetative stage were attacked by several organisms, both insect herbivores and plant pathogens. The parasitoid species used parasitize the caterpillars (*Cotesia glomerata*) or the eggs (*Trichogramma brassicae*) of *Pieris brassicae,* or *Brevicoryne brassicae* aphids (*Diaeretiella rapae*). Photos credited to bugsinthepicture.com, and more specifically Nina Fatouros (*T. brassicae* and *D. rapae*), Hans Smid (*C. glomerata*) and Tibor Bukovinszky (*P. brassicae* eggs).

Insect herbivores

For the insect herbivores, the work in this thesis focused on two species which frequently co-occur on brassicaceous plant species in nature. Caterpillars of the large cabbage

white butterfly, *P. brassicae*, and cabbage aphids, *B. brassicae*, are both specialist herbivore species of brassicaceous plants, including *B. nigra*. Butterflies of *P. brassicae* lay eggs in large clutches (30-100 eggs), and caterpillars feed gregariously during the first four of the five larval stages. As voracious feeders, *P. brassicae* caterpillars pose a major threat to plants as they are capable of entirely defoliating them. The egg stage was also chosen to be included as it has been shown that egg deposition can be sensed by the plant, triggering defense responses (Bruessow *et al.*, 2010) and affecting both plant volatile emission and the foraging behavior of the larval parasitoid of *P. brassicae*, *Cotesia glomerata* (Fatouros *et al.*, 2012; Pashalidou *et al.*, 2014).

Plant pathogen

The bacterial pathogen *Xanthomonas campestris* pv. *campestris*, commonly known as black rot, is one of the major, and most devastating pathogens to infect brassicaceous crops, causing substantial economic losses worldwide. The bacteria develops optimally in warm (25-30°C) humid (80-100%) conditions, though infected plants can remain symptomless under 18°C. Plants of any age, including seedlings, are infected via natural openings, such as hydathodes, or to a lesser extent, stomata, or through wounds on the leaves or roots. Initial symptoms are characterized by small v-shaped chlorotic to necrotic lesions on the leaf margins, which gradually spread inwards (Fig. 2). Veins turn black as the pathogen spreads through the vascular tissue and once the stem is attained, the bacteria then spread to other portions of the plant, potentially leading to its complete demise.

Typically, plants of the Rhine population of *B. nigra* used in this study display moderate disease symptoms, i.e. yellowing and necrosis around the infiltration sites, with little spread to other parts of the plant, which indicates a degree of tolerance to the specific strain used. Spreading to other portions of the plant, if any, becomes evident 5-8 days post infection. However, the presence and severity of disease symptoms is also dependent on pathovar used. In this thesis two pathovars were included (Chapter 5), the common *X. campestris* pv. *campestris* described above, and *X. campestris* pv. *incanae*, which is also specific to brassicaceous plants.

Plant interactions with pathogenic microbes can be of one of two types: either compatible, where a pathogen successfully invades a host plant, or incompatible, where the pathogen fails to infect the plant. *Xanthomonas campestris* pv. *campestris* is considered to be virulent on *B. nigra* as it leads to symptom development, while *X. campestris* pv. *incanae* is considered avirulent on *B. nigra*, i.e. the plants successfully defend themselves, which is manifested by the presence of hypersensitive response lesions (cell death at the site of pathogen entry

which aims to inhibit further colonization of the plant tissues) at the site of infiltration.

Parasitoid wasps

The behavior of three different parasitoid species was studied in this thesis, that either parasitized the *B. brassicae* aphids, or the egg or larval stages of *P. brassicae*. *Diaeretiella rapae* is a generalist aphid parasitoid, however it is the main primary solitary endoparasitoid of *B. brassicae* (Blande *et al.*, 2004; Bukovinszky *et al.*, 2008) Wasps lay a single egg into an aphid and the parasitoid larvae develops inside the aphid, eventually killing it and turning the aphid corpse into a mummy. *Cotesia glomerata* is a gregarious larval endoparasitoid, with their main host being *P. brassicae* caterpillars. Females lay between 10 and 40 eggs within each host caterpillar, generally young first or second instar caterpillars, while allowing the host to continue to develop normally after being parasitized (=koinobiont host interaction). Parasitoid larvae emerge from the host just prior to pupation, and spin their cocoons in close proximity to their dying host. *Trichogramma brassicae* is a minute wasp species, parasitizing the eggs of various lepidopteran species, including *P. brassicae*. Females lay one or more eggs inside the host egg, and as the larvae develops, the egg dies and turns black.

Thesis outline

In **Chapter 2**, the influence of attack by insect herbivores and/or phytopathogens on defense signaling pathways and induced plant volatiles is reviewed and discussed. While herbivore-induced plant volatiles are well studied, only limited knowledge is available on the induction of plant volatiles by more than one attacker, and these studies are often restricted to investigating the effects of multiple insect herbivores on volatile-mediated foraging behavior of single parasitoid species. In addition to herbivory, plant pathogens represent a major and omnipresent threat to plants, yet the effect of these on plant volatiles and their ensuing utilization by organisms associated with these plants, such as natural enemies of the insect herbivores, has been largely overlooked. In this chapter I bring together the evidence demonstrating the strong impact that insect herbivores, plant pathogens or a combination of the two can exert on plant volatile emissions and tritrophic interactions, and underline the commonalities that may exist between herbivore- and pathogen-induced volatile blends.

Chapter 3 investigates the effects of three different non-host attackers, alone or in combination with *P. brassicae* host caterpillars, on the foraging behavior of *C. glomerata*

parasitoid wasps. Here, I investigated the attraction of *C. glomerata* to plant volatiles when the plant is challenged by insect herbivores or a phytopathogen, both alone and in combination with the parasitoids' caterpillar hosts. In addition to behavioral studies, I chemically analyzed the volatile blends emitted by plants infested by single or combinations of attacker species to determine how dual attack modified the blends, and to link changes in the blend to the observed parasitoid behavior. As the non-host attackers (eggs, aphids or pathogen infection) and host attackers (caterpillars) theoretically induce plant defenses via opposing defense signaling pathways, we expected the resulting 'crosstalk' to impact on volatile-mediated foraging.

In **Chapter 4,** a complementary approach to Chapter 3 is taken by investigating the behavioral response of three different parasitoid species to volatiles emitted by plants dually challenged by host and non-host herbivores. Here the effects of dual infestation with *B. brassicae* aphids and *P. brassicae* eggs or caterpillars on induced volatiles and respective parasitoid (*D. rapae*, *T. brassicae* and *C. glomerata*) foraging was studied more closely. More specifically, I assessed if the effects of dual herbivory were comparable across the three different parasitoid species, and investigated the importance of aphid density during dual attack.

Chapter 5 delves deeper into the effects of plant pathogen infection on tritrophic interactions. I further addressed the impact of pathogens by comparing the effect of both a virulent (disease causing) and an avirulent (plant resistance) *X. campestris* strain on host-induced volatiles and wasp foraging, as plant-pathogen compatibility is known to lead to different plant responses. I used caterpillars of *P. brassicae* as hosts and *C. glomerata* wasps as the focal interaction.

Chapter 6 provides a detailed look at the metabolome of *B. nigra* plants challenged by *P. brassicae* caterpillars and two densities of *B. brassicae* aphids, in order to investigate if the density-dependent effects found in earlier chapters were also present at the sub-cellular level. The metabolome was analyzed from a global perspective, assessing changes in both the local, infested leaves and the adjacent systemic leaves, and effects in both primary and secondary metabolism were explored.

Finally, in **Chapter 7**, the findings of this thesis are integrated and discussed, with a focus on the key aspects to consider in research on plant responses to multiple attack, and present an outline of the future directions that may allow a greater understanding of the role of plant volatiles in the foraging behavior of natural enemies under more realistic multiple attack scenarios.

Acknowledgements

I thank Rieta Gols and Marcel Dicke for their constructive comments on earlier versions of this chapter.

Chapter 2

Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens

Camille Ponzio [1], Rieta Gols [1], Corné M.J. Pieterse [2] & Marcel Dicke [1]

[1] Laboratory of Entomology, Wageningen University, P.O. Box 16, 6700 AA Wageningen, The Netherlands

[2] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, The Netherlands

Published in Functional Ecology, 27(3), 587-598 (**2013**)

ABSTRACT

In their natural environment plants are faced with a multitude of attackers, of which insect herbivores and plant pathogens are an important component. In response to these attacks, plants release volatile organic compounds (VOCs), which play an important role in the communication between plants and the associated community members, such as other herbivores, phytopathogens and the natural enemies of herbivores. While numerous studies have focused on either plant-pathogen or plant-insect interactions, less is known of interactions where these two sets of interactions co-occur. Depending on the mode of attack of the pathogen (necrotroph vs. biotroph) or herbivore (chewing vs. piercing-sucking) they will activate different defense pathways in the plant in which the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play key roles. As these pathways can crosstalk, pathogen infection can interfere in a plant's defense response to herbivory, and vice versa. Infestation of a plant with organisms inducing SA signaling prior to - or simultaneously with- attack by organisms that induce the JA pathway often suppresses JA signaling. However, the impact of this signaling pathway crosstalk on VOC induction is not clear cut, as there is high variability in the effects on volatile emissions, ranging from suppression to enhanced emission. The effects of the modified volatile blends on the foraging success of carnivorous natural enemies of herbivorous insects have started to be investigated. Foraging success of natural enemies generally withstands this modification of the host-induced VOC blend, but the presence or absence of key compounds is an important determinant of the response of certain carnivores. Further studies incorporating plant-insect and plant-pathogen interactions at different levels of biological integration will provide valuable insight in how plants integrate signals from different suites of attacking organisms into an adaptive defense response.

Keywords: tripartite interactions, tritrophic interactions, signal-transduction, phytohormones, species interactions, natural enemies, plant volatiles

Introduction

In nature, plants are members of complex communities and they interact with a wide range of organisms, both beneficial and deleterious. Among these are a plethora of attackers, including herbivorous arthropods and plant pathogens. Half of the estimated 6 million insect species are herbivorous (Schoonhoven *et al.*, 2005) and while the diversity of plant pathogenic microbes has not been quantified, they are an equally major threat to plants (Strange & Scott, 2005). Faced with this multitude of enemies, it should come as no surprise that plants have evolved sophisticated defense strategies that allow them to recognize herbivores or pathogens (Mithöfer & Boland, 2008) and then implement an often tailor-made defense response. When a plant is under attack, a wide range of responses are initiated, including physical and chemical defenses (Walters, 2011). The latter of these include the production of secondary metabolites (Iason *et al.*, 2012) of which the emission of volatile organic compounds (VOCs) are an important component (Heil, 2008; Dicke & Baldwin, 2010; Hare, 2011).

VOCs are highly diverse and are of strong ecological importance as they contribute to shaping the assemblage of, and interactions between, the organisms within a plant's community (Poelman *et al.*, 2008b). VOCs induced by herbivory attract natural enemies of herbivores, which may confer protection to the plant (Kessler & Baldwin, 2001; Dicke & Baldwin, 2010). Induced volatiles can have repellent effects on herbivores (Delphia *et al.*, 2007; Bleeker *et al.*, 2009) and inhibit pathogen colonization (Brown *et al.*, 1995). In addition, they are involved in information transfer between and within plants, through the airborne transport of signals which lead to the priming or expression of defenses in neighboring plants or in distal parts of the emitting plant (Engelberth *et al.*, 2004; Kessler *et al.*, 2006; Frost *et al.*, 2008). These VOCs, however, can be a double-edged sword as they can also be used by herbivores as host-plant location cues (Bolter *et al.*, 1997). For instance, in *Nicotiana attenuata*, the same volatile chemical signals are exploited by both herbivores and carnivores for host location (Halitschke *et al.*, 2008) underlining the complexity in the interactions mediated by VOCs (Dicke & Baldwin, 2010).

While induced VOCs are receiving increasing attention, especially their role in mediating tritrophic interactions, the study of VOCs in a multiple attack situation, notably with pathogens and herbivores, has been largely unexplored. In nature, plants are often confronted with simultaneous or sequential attack, yet even until recently research was largely conducted on study systems comprising of single plant-attacker combinations (Dicke *et al.*, 2009). Rapid advances in our knowledge on the underlying mechanisms of plant defenses have shown that the interactions under a multiple attack scenario

are complex (Pieterse *et al.*, 2012). At least three phytohormones, i.e. salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), play key regulatory roles in the interconnecting signal-transduction pathways which mediate induced defenses in response to herbivore and pathogen infestation. Crosstalk between these pathways can mold the final defense response, including VOC production, when challenged by different attacker species. Induced responses of plants to pathogens and herbivores were long treated as two separate fields, and the two areas of study have evolved largely independently of one another until quite recently (Pieterse & Dicke, 2007). In this review, we will give an overview of (1) the existing literature on crosstalk between signaling pathways affected by insect herbivore and pathogen attack, (2) the effect of pathway crosstalk on plant VOC emission, and (3) the effects of these emissions on community members at higher trophic levels. Finally, we will provide an outlook to the future by focusing on the integration of studies on plant-pathogen and plant-insect interactions.

Signal-transduction pathways regulating induced plant defenses

At the crux of a plant's interactions with attacking pathogens and insect herbivores lays a complex interconnecting signaling network regulated by phytohormones. These are crucial not only for regulating plant growth, development and reproduction, but also for induced defenses, including the production of VOCs. It has been well established that SA, JA and ET play pivotal roles in the regulation of the signal-transduction pathways that lead to the activation of different sets of defense-related genes (von Dahl & Baldwin, 2007; Howe & Jander, 2008; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). While the common division is that pathogens induce SA and herbivores activate JA-mediated defenses, the reality is much more complex. For example, SA is generally thought to mediate defenses against piercing-sucking insects and pathogens which are biotrophic for all or part of their lifecycle, while JA/ET induction is usually associated with chewing insect herbivores and necrotrophic pathogens (Glazebrook, 2005; Pieterse & Dicke, 2007; Spoel *et al.*, 2007). Moreover, defenses against herbivores and necrotrophs are seemingly regulated by two distinct and antagonistic branches of the JA signaling pathway (Kazan & Manners, 2008; Verhage *et al.*, 2011) (Fig. 1). In addition, plant-attacker combinations of organisms inducing the same general pathways generate different dynamics of SA, JA and ET production and, consequently, different transcriptomic responses (De Vos *et al.*, 2005). This unique 'signal signature' induced by an attack results in major differences in the expression levels and timing of the different gene sets, contributing to the specificity of a plant's induced response which is likely to have important consequences for the

volatile blends induced by the signal-transduction pathways.

In nature, plants often face multiple attackers and they need to be able to adapt to their ever-changing environment. There is increasing evidence that signaling pathways cross-communicate with each other, and it is clear that this crosstalk is a powerful regulatory mechanism that allows plants to fine-tune their final defense response in reaction to pathogen or herbivore attack (Koornneef & Pieterse, 2008; Grant & Jones, 2009; Verhage *et al.*, 2010). The outcome of crosstalk between different signal-transduction pathways can be antagonistic or synergistic and the interaction between the SA and JA signal-transduction pathways is one of the best studied examples, with much evidence that they are often mutually antagonistic (Stout *et al.*, 2006; Koornneef *et al.*, 2008; Koornneef & Pieterse, 2008). SA has an especially strong effect, in that its accumulation inhibits JA biosynthesis and signaling when it is induced prior to or concomitantly with JA. In Arabidopsis, infection by the hemi-biotroph *Pseudomonas syringae* leads to greater susceptibility to the nectrotroph *Alternaria brassicicola*, through impairment of JA-mediated defenses (Spoel *et al.*, 2007). ET is involved in crosstalk with both of these pathways, as it acts synergistically with JA in activating defense-related genes (Penninckx *et al.*, 1998)1998 and can heighten a plant's sensitivity to SA, leading to enhanced SA-mediated defenses (De Vos *et al.*, 2006). Other phytohormones, such as abscisic acid, auxins, cytokinins, brassinosteroids and gibberellins appear to play a much larger role in shaping defense-related signaling than previously thought, although for some the exact mechanisms of their involvement in the backbone SA-JA-ET network still needs to be further explored (Erb *et al.*, 2011a; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Giron *et al.*, 2013). In the context of multiple attackers, prioritization of one pathway over another can have strong consequences in terms of defense expression. Order of attack becomes important as the initial attack may compromise a plant's induced responses to secondary challengers, driving community-wide effects (Bruinsma & Dicke, 2008; Poelman *et al.*, 2008b). At the molecular level, it has also been demonstrated that timing of induction is a major factor determining JA and SA signaling (Thaler *et al.*, 2002; Koornneef *et al.*, 2008).

Interestingly, dose-dependent effects have been suggested to influence JA-SA crosstalk. Low concentrations of both exogenous SA and JA enhance the expression of defense-related genes, leading to a synergistic effect. However, at higher concentrations or prolonged treatment duration this effect disappears, indicating that there may be threshold levels for defense trade-offs (Mur *et al.*, 2006; Kazan & Manners, 2008). Recent studies show an important modulatory role of ET in crosstalk in that when JA/ET is induced first, the ET burst renders JA-dependent defenses insensitive to SA-mediated suppression (Diezel *et al.*, 2009; Leon-Reyes *et al.*, 2010). Herbivores and pathogens have evolved

ways of manipulating hormonal crosstalk to their own advantage, and so can change the outcome of defense. A well-known example is *P. syringae* which produces coronatine, a JA analogue that suppresses SA-dependent defenses, rendering plants more susceptible to the pathogen (Nomura *et al.*, 2005). More recently, similar hijacking mechanisms were found for a necrotrophic fungus, *Botrytis cinerea* (El Oirdi *et al.*, 2011). Similar decoy tactics exist among insects, such as in the case of *Bemisia tabaci* nymphs that promote their own development as well as spider mite reproduction by down-regulating JA defenses via SA induction (Zarate *et al.*, 2007; Zhang *et al.*, 2009). The spider mite *Tetranychus evansi* takes this one step further by not only preventing defense induction, but also suppressing the constitutive plant defenses, which enhances the performance of conspecifics (Sarmento *et al.*, 2011).

Single species herbivory

When a plant is attacked by an herbivore, this induces the emission of a specific blend of volatile compounds, known as herbivore-induced plant volatiles (HIPV) (Fig. 1). HIPV are complex blends that can be composed of up to several hundred individual compounds (Pichersky & Gershenzon, 2002; Dudareva *et al.*, 2006; Pichersky *et al.*, 2006). These volatile blends are generally dominated by two major classes of compounds: terpenoids and fatty acid derived green leaf volatiles (GLV) (Arimura *et al.*, 2009; Mumm & Dicke, 2010). Some compounds, primarily GLV, are mainly released immediately upon wounding while others, including terpenoids, are synthesized 'de novo' and released from several hours up to several days after attack (Paré & Tumlinson, 1997; Turlings *et al.*, 1998). Many HIPV are not induced by mechanically damaging tissues alone, and in many cases they will be emitted both locally from damaged leaves and systemically from undamaged plant tissues. Some compounds may already be produced by undamaged plants and are simply emitted in greater quantities or different ratios after herbivory (Holopainen, 2004). HIPV blends can vary quantitatively and qualitatively, depending on the plant species (Takabayashi *et al.*, 1991; van Poecke & Dicke, 2004), plant genotype (Degen *et al.*, 2004; Kappers *et al.*, 2011), herbivore species (De Moraes *et al.*, 1998) and even the developmental stage of herbivores (Takabayashi *et al.*, 1995).

Disparities in volatile responses to different herbivore species can be in part explained by differences in the damage inflicted by insects of contrasting feeding guilds, due to the distinctive signaling pathways that they induce (Leitner *et al.*, 2005). However, as even insects of the same guild can induce different HIPV blends, variation in volatile profiles can also be attributed to the composition of elicitors present in oral secretions

of herbivores (Halitschke *et al.*, 2003; Tumlinson & Engelberth, 2008). Application of oral secretions, or of elicitors present in herbivore regurgitant, to wounded leaf tissue can mimic the effects of herbivory and activate the signal-transduction pathways, leading to the biosynthesis and emission of HIPV (Turlings *et al.*, 1990; Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Bonaventure *et al.*, 2011).

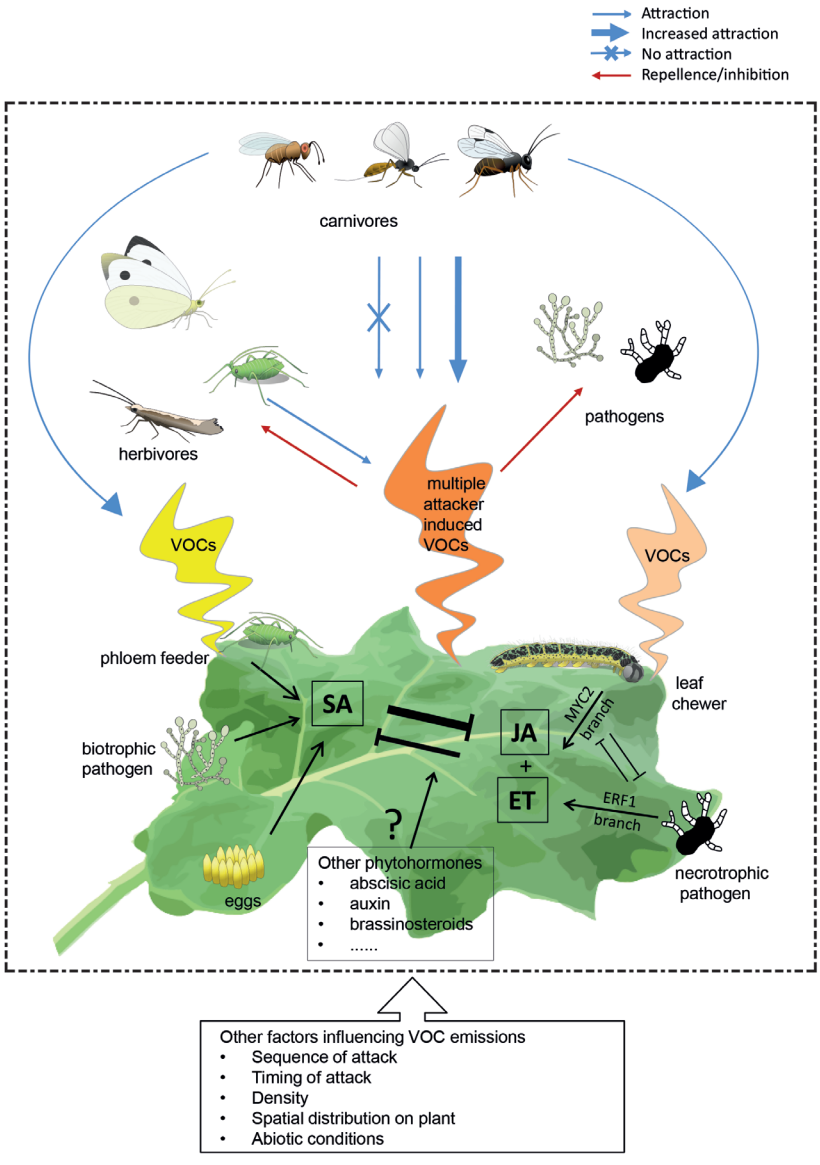


Figure 1. Overview of the effects of single and multiple attack of plants on VOC emission, including subcellular mechanisms such as phytohormone-mediated signal-transduction and effects on interactions with microbial and insect species.

Further complicating matters is that larval feeding is usually preceded by oviposition, which is a fact that has been long neglected in studies on HIPV. Insect egg deposition can also lead to the induction of volatile emission, which then mediates the attraction of egg parasitoids (Hilker *et al.*, 2002; Mumm *et al.*, 2003; Fatouros *et al.*, 2008; Tamiru *et al.*, 2011). In the pine and elm systems, elicitation of volatiles attractive to the parasitoids was shown to be a response to compounds in the oviduct secretions coating the eggs, which then come in contact with leaf tissue during oviposition (Meiners & Hilker, 1997; Hilker *et al.*, 2002). In other cases, egg parasitoids respond to volatiles that are only induced by the combination of both feeding damage and oviposition, as oviposition or feeding alone were not attractive to parasitoids (Colazza *et al.*, 2004a; Colazza *et al.*, 2004b). The presence of both feeding damage and eggs can also have a synergistic effect on the emissions of certain compounds (Conti *et al.*, 2008). However, Bruessow *et al.* (2010) found, unexpectedly, that egg deposition by *Pieris brassicae* butterflies leads to the local accumulation of SA, which then suppresses expression of caterpillar-induced JA-dependent defense genes. This crosstalk can have important consequences for plant defense, and there is evidence that HIPV emissions normally induced by subsequent caterpillar herbivory can be suppressed (Penaflor *et al.*, 2011). From a mechanistic perspective, looking at different stages of the plant-herbivore interaction independently is a suitable approach. However, these studies demonstrate that it is imperative to investigate the sequence of events as they occur in nature, with eggs first, in order to have an accurate overview from an ecological point of view.

Multiple herbivory

There are an increasing number of studies which investigate the dynamics of plant-insect interactions under multiple attack. However, many of these focus primarily on how direct defenses mediate interactions between herbivores (Rodriguez-Saona *et al.*, 2005; Kaplan & Denno, 2007). Knowing that there is potential for crosstalk between the SA and JA signaling pathways in particular, it is likely that interactions between attackers of different feeding guilds can also affect induction of indirect plant defenses. Attack by an SA-pathway-inducing herbivore can be expected to modify or attenuate the volatile response toward a subsequent JA-pathway-inducing herbivore, and vice-versa.

What is clear from the literature to date is that the effect of dual herbivory on HIPV emissions is difficult to predict and highly variable. Considering feeding guilds alone is not enough to make predictions, as the same combination of herbivore species may have drastically diverging effects on ensuing VOC emissions in different plant species (De

Boer *et al.*, 2008). Multiple herbivory by insects of different feeding guilds can result in a few specific compounds being emitted in significantly higher or lower amounts than by attack by one herbivore only, with an overall volatile profile similar to what is induced in singly damaged plants (Rodriguez-Saona *et al.*, 2003; Delphia *et al.*, 2007; Zhang *et al.*, 2009). In other instances, total emission rates are affected. Dual herbivory involving different feeding guilds can lead to the majority of compounds being emitted in greater amounts than during single species herbivory (Moayeri *et al.*, 2007; Hare & Sun, 2011) and HIPV emissions may also increase to levels higher than the expected additive effect of the two herbivores, indicating a synergistic effect on induction (De Boer *et al.*, 2008). Total emissions can also be suppressed, as was demonstrated in cotton plants infested by piercing silverleaf whiteflies (*B. tabaci*) and chewing *Spodoptera exigua* caterpillars, where HIPV emissions in response to dual herbivory were reduced by 60% in comparison to caterpillar feeding alone (Rodriguez-Saona *et al.*, 2003). Finally, in still other cases, damage by a phloem feeder did not lead to any measurable effects on HIPV emissions induced by caterpillars (Erb *et al.*, 2010). Moreover, changes in HIPV emissions are not limited to those brought about by two folivorous herbivores, but also by combinations of shoot and root herbivory, which can also lead to altered patterns of emission in one or both spheres (Rasmann & Turlings, 2007; Soler *et al.*, 2007; Pierre *et al.*, 2011).

One trend that emerges from the literature is that when an herbivore, usually a phloem feeder, induces low HIPV emissions, volatile emission elicited by a co-attacking chewing herbivore can be negatively affected (Rodriguez-Saona *et al.*, 2005; Zhang *et al.*, 2009; Schwartzberg *et al.*, 2011). Although not a phloem feeder, maggots of the gall-inducing tephritid fly, *Eurosta solidaginis* attacking *Solidago altissima*, had similar effects, in that they dampened total HIPV production normally induced by *Heliothis virescens* caterpillars. Interestingly, two other herbivores that did not induce HIPV, i.e. *Gnorimoschema gallaesolidaginis* (galling moth) and *Philaenus spumarius* (piercing-sucking insect), failed to have the same suppressive effect on *H. virescens*-induced volatiles. However, researchers were unable to clearly establish the role of the primary defense-related phytohormones, in part due to high variability within the dataset (Tooker *et al.*, 2008).

Of all the multiple herbivory studies specifically addressing HIPV, few have simultaneously investigated the phytohormonal basis for the observed changes in HIPV production. Zhang *et al.* (2009) observed that whitefly (*B. tabaci*) infestation negatively affected the emission of a spider-mite (*Tetranychus urticae*) induced monoterpene and this interference was positively damage-dependent. JA levels in dually infested leaves were significantly lower compared to levels in leaves infested with *T. urticae* only, and two JA-regulated genes involved in induced defense signaling and the terpenoid's biosynthesis showed reduced

expression levels. Application of exogenous SA mimicked these results, indicating that the interference of *B. tabaci* in volatile emission may be mediated by the SA signaling pathway. In another study, attack by the phloem feeder *Euscelidius variegatus* did not alter the volatile blend induced by *Spodoptera littoralis* caterpillars, suggesting an absence of negative crosstalk in this system (Erb *et al.*, 2010). Although no phytohormonal analyses have been made in the latter study, transcriptional data supports this suggestion, as it showed that contrary to many phloem feeders, *E. variegatus* induced the same JA biosynthetic genes as *S. littoralis*. However, it remains to be investigated whether the effect is dependent on the amount of damage.

VOC induction by pathogens

Plant pathogens are also capable of inducing plant volatiles, although this has been far less studied than induction by herbivorous insects (Fig. 1). As with herbivores, the induced VOC blend can exhibit attacker specificity, with different strains of a same pathogen inducing quantitatively and qualitatively differing VOC blends (Huang *et al.*, 2003). In comparison to HIPV, the ecological function of pathogen-induced plant volatiles is not very clear yet, though it is thought that they may function as an additional defense mechanism against pathogen attack. Volatile emission from infected plants has been found to correlate with a hypersensitive response (local cell death in the region surrounding the infection site) in the plant (Croft *et al.*, 1993; Huang *et al.*, 2003). Furthermore, several pathogen-induced volatile compounds have been shown to severely inhibit pathogen growth. Such antimicrobial activity was shown for compounds such as (*Z*)-3-hexenol and (*E*)-2-hexenal (Croft *et al.*, 1993) as well as for methyl salicylate (MeSA) and linalool (Shulaev *et al.*, 1997; Cardoza *et al.*, 2002), which are compounds that are also often emitted in response to herbivory.

The mechanisms underlying volatile production by plants in response to pathogen infection are not well studied, particularly in relation to the phytohormonal signaling pathways. Several microbial elicitors of VOC induction have been identified, and interestingly it appears that some pathogens induce similar patterns of VOCs as those induced by herbivory. One example of this is cellulysin, derived from the fungus *Trichoderma viridae,* which was found to induce volatiles via the JA signaling pathway (Piel *et al.*, 1997). Interestingly, alamethicin, isolated from the same fungus, induced only a few volatile compounds; two homoterpenes, whose biosynthesis is dependent on the JA signaling pathway, and MeSA (Engelberth *et al.*, 2001). The implication of phytohormones

in volatile induction is not so straightforward, as was recently shown. High accumulation of JA does not systematically lead to VOC induction. The application of two microbial elicitors, β-(1,3)-β-(1,6)-glucans and *N,N',N'',N'''*-tetra acetylchitotetraose, both give rise to an accumulation of JA 8-fold higher than levels observed during herbivory. Despite high JA levels, only the elicitor β-(1,3)-β-(1,6)-glucans led to substantial VOC induction (Leitner *et al.* 2008). This indicates that induction of the JA signaling cascade is not solely involved in triggering volatile induction in this plant species and that other mechanisms, yet to be determined, are likely to be implicated as well. As phytopathogen infection may result in specific plant responses in terms of VOC emissions, the influence of phytopathogens, alone or in combination with an insect herbivore, on VOC induction in plants, as well as the unravelling of the underlying signaling pathways, deserve more attention.

Induced plant VOCs in interactions between phytopathogens and insect herbivores

A number of studies have examined indirect plant-mediated interactions between pathogens and herbivores, primarily in terms of attacker performance. The outcome of such interactions is dependent on the plant, herbivore and pathogen species involved. Pathogen infection may have either beneficial, neutral or detrimental effects on herbivore performance, and likewise herbivory may affect pathogen growth (for a comprehensive overview see Rostás *et al.*, 2003). However, despite abundant literature, crosstalk effects between phytohormonal signaling pathways and the impact of pathogens on plant volatile emissions remain largely unexplored.

Plant volatiles induced by pathogen infection alone can influence interactions between a plant and its herbivores. These volatile emissions can make the plant more attractive to herbivores (Piesik *et al.*, 2011) and can also be used by female insects to discriminate against infected plants for oviposition (Dötterl *et al.*, 2009; Tasin *et al.*, 2012) and even differentiate between VOC profiles induced by two different pathogenic fungi (Johne *et al.*, 2008).

The very limited number of studies that have examined plant responses to dual stresses showed that co-occurring attack by a pathogen and an herbivore affects the plant's VOC emissions in response to herbivory, and effects are consistent with expectations in the light of knowledge of underlying phytohormonal signal-transduction. Concomitant attack by a hemibiotrophic fungus, *Setosphaeria turcica,* and a leaf chewing herbivore, *S.*

exigua, resulted in strongly suppressed volatile emissions in comparison to herbivory alone (Rostás *et al.*, 2006). Classically categorized as a necrotroph, recent work suggests that *S. turica* is in fact a hemibiotroph (Chung *et al.*, 2010), and the plant defense response to the fungus in the early infection stages appears to be SA and ET pathway regulated (Erb *et al.*, 2009). In light of this, the reduced volatile emissions may be an effect of crosstalk with herbivore-induced JA-dependent defenses. A similarly attenuated volatile emission (60% reduction) was reported for dual attack by a leaf chewer and a phloem feeder (Rodriguez-Saona *et al.*, 2003), and it is generally accepted that phloem feeders and (hemi-) biotrophic pathogens elicit similar responses in the plant (Walling, 2000). In contrast, infection of peanut plants by the necrotrophic pathogen *Sclerotium rolfsii*, thought to primarily induce JA, had no negative effect on VOC emissions in response to herbivory by *S.exigua* caterpillars. Along with pathogen-induced volatiles, the plants emitted all the compounds that are produced in response to *S. exigua* herbivory alone, often in greater amounts (Cardoza *et al.*, 2002). Dual induction of the JA signal-transduction pathway may explain the enhanced VOC emissions. Cardoza *et al.* (2003a) later showed for this system that dually challenged plants had significantly higher levels of JA than the expected additive effect of the individual attackers. The nature of the interaction between a plant and pathogen strain can add a further layer of complexity to the outcome of the plant-pathogen-insect interaction. A plant's volatile response to herbivory can substantially differ if the co-occurring strain of *Xanthomonas campestris* has either a compatible (infection) or incompatible (plant resistance) interaction with the plant. Compared to herbivory alone, co-attack by a compatible strain enhanced VOC emissions, while infection with an incompatible strain led to the suppression of herbivore-induced compounds (Cardoza & Tumlinson, 2006).

Volatile emissions can also be manipulated by pathogens to their own benefit, namely the attraction of insect vectors in order to ensure dispersal to other host plants. This is commonly the case with viruses, which can alter plant volatile emissions resulting in attraction of aphid vectors to plants which may even be of suboptimal quality for them (Fereres & Moreno, 2009; Bosque-Pérez & Eigenbrode, 2011), or decrease the attraction of non-vectoring herbivores (van Molken *et al.*, 2012). Virus infection can lead to elevated levels of the same volatile blend produced by healthy plants (Eigenbrode *et al.*, 2002; Mauck *et al.*, 2010) which can make infected plants more attractive to herbivores. Such manipulation of plant volatiles is not restricted to viruses. Both bacterial (Mayer *et al.*, 2008) and fungal pathogens (McLeod *et al.*, 2005) can induce changes in their host plant's volatile emissions that result in the attraction of insect vectors, which will then carry the pathogen to new host plants.

Effects of VOCs on the natural enemies of herbivores

Many of the studies on HIPV in the context of tritrophic interactions were conducted with one plant, herbivore and carnivorous species. However, even in these simple systems, it is apparent that there exists a large amount of variation in HIPV induction between organisms and study systems. Moreover, our knowledge on the mechanisms of defense induction indicates that simultaneous or sequential attack by different herbivore species, particularly when they belong to different feeding guilds, can have important consequences for the ecological dynamics of a tritrophic system (for a review see Poelman *et al.*, 2008b; Dicke *et al.*, 2009). It is widely recognized that carnivorous insects exploit plant volatiles that are produced in response to feeding damage as a navigational system for prey/host location, and much attention has been given to the roles of induced VOCs in mediating tritrophic interactions (Sabelis & Van De Baan, 1983; Turlings *et al.*, 1995; Heil, 2008; Dicke & Baldwin, 2010; Reddy, 2012).

The effects of multiple attack on natural enemies foraging success can be positive, negative or neutral. For instance, when two organisms inducing the JA signaling pathway co-occur on a plant, natural enemies often become more attracted to such plants (Shiojiri *et al.*, 2001; De Boer *et al.*, 2008), also when the second attacker is a pathogen (Cardoza *et al.*, 2003b). However, this may depend on the natural enemy species. Shiojiri *et al.* (2001) showed that herbivory by *Plutella xylostella* and *Pieris rapae* caterpillars enhanced attraction of the parasitoid *Cotesia glomerata* even though it can only parasitize one of the lepidopterans, while *C. plutellae* preferred VOCs of plants infested with only its host. On dually infested plants this then translates into increased *C. glomerata* parasitism rates, and a decrease for *C. plutellae*, than on plants with only their respective hosts (Shiojiri *et al.*, 2002). Some studies have also examined effects on the third trophic level when both the JA and SA signal-transduction pathways are assumed to be induced, by combinations of herbivores and/or pathogens. While in one case attack by two herbivore species respectively affecting JA and SA signaling led to a decrease in carnivore attraction compared to single-species herbivory (Zhang *et al.*, 2009), in all other cases carnivores were either equally attracted to, or preferred dually damaged plants over plants damaged by the host herbivore alone, for both dual attack by two herbivores (Moayeri *et al.*, 2007; Erb *et al.*, 2010) and a combination of herbivore and pathogen (Rostás *et al.*, 2006). Interactions between the two primary signal-transduction pathways may play a role in the changes in VOC emissions in response to dual attacker events. This in turn may affect the response of natural enemies depending on the blend characteristics a species uses in VOC-mediated foraging behavior.

While higher levels of VOC emissions can increase the attraction of natural enemies in both single and multiple attack scenarios, changes in levels of specific compounds after dual attack can have serious implications for foraging decisions. Absence of a key compound can lead to a greatly diminished response of a predator. Predatory mites showed a strongly diminished response to plants infested by spider mites and non-prey whiteflies (Zhang *et al.* 2009). Dually damaged plants were shown to have reduced levels of (*E*)-β-ocimene emission, which is a compound known to be attractive to the predator (Dicke *et al.*, 1990). Supplementing the blend from spider-mite plus whitefly infested plants with this compound restored predator attraction, demonstrating its importance for locating prey by the predators (Zhang *et al.*, 2009). The reduction in (*E*)-β-ocimene emission in response to whitefly infestation correlated with reduced JA titre and reduced transcription of the JA-regulated gene encoding for the enzyme ocimene synthase that is crucial for in (*E*)-β-ocimene production (Zhang *et al.* 2009). Likewise, a sharp decrease in overall volatile emissions may not have any consequences for parasitoid attraction if the compounds involved in attraction remain relatively unaffected in their emission rates (Rostás *et al.*, 2006). Thus, in order to understand the consequences of dual infestation on the behavioral responses of carnivorous insects, knowledge of the underlying mechanisms is crucial.

Conclusions and future perspectives

In natural systems, co-occurring attack by herbivorous insects and phytopathogens is frequent, and there is a tendency to increase the complexity of the studies investigating plant defense in order to more realistically reflect natural conditions. Yet, while plant-mediated effects of plant pathogens on insect herbivores, and vice versa, have been examined in some detail, e.g. in terms of attacker performance, their combined effects on underlying signal-transduction networks and induced volatile responses have only rarely been considered. Recent studies show that including another insect attacker greatly increases the complexity of interactions between the plant and its attackers, both in terms of direct and indirect plant-mediated defenses (Dicke *et al.* 2009), so the inclusion of phytopathogens into the system can be expected to have similar effects. The very limited research on the subject so far indicates that not only are phytopathogens capable of inducing plant volatiles, sometimes similarly to the response to herbivory, they can also have an influence on host-plant searching behavior of herbivores, and also on insects of the third trophic level which often rely on volatile cues for locating their herbivorous victims. This topic warrants further research, as the combination of plant-

insect and plant-pathogen interactions into one system will provide important insight into how plants prioritize and integrate signals coming from different suites of attacking organisms.

Our knowledge of VOC induction under multiple attack is still too limited to be able to draw solid conclusions about how plants determine VOC induction patterns, Even in the more intensively studied area of HIPV it is apparent that the knowledge of what happens during single herbivory is not sufficient to predict a plant's responses when facing multiple herbivores (Dicke *et al.*, 2009). Dual attack appears to lead to high variability in the effects on VOC emission; several factors influencing signal-transduction networks and subsequent VOC induction (below) must be taken into consideration in order to reveal emerging patterns.

Attacker identity. Different combinations of attacking organisms may induce different signaling pathways, so the final plant defenses will be highly dependent on the combination used. Induction with two organisms that induce the same defense pathways will have different effects than two organisms inducing different pathways.

Severity of attack. Plant responses to herbivory, both in single and in multiple herbivore attack are dependent on the amount of damage inflicted by attacking herbivores (Geervliet *et al.*, 1998; Zhang *et al.*, 2009). It is likely that the severity of pathogen infection also exhibits dose-dependent effects on volatile emissions.

Sequence of attack. The order in which organisms attack can be an important determinant of the plant's response (Viswanathan *et al.*, 2007; Erb *et al.*, 2011b), and SA-mediated induction prior to - or simultaneously with - JA-mediated induction is most often researched. Though the inhibitive effects of SA on JA are stronger than the contrary (Koornneef *et al.*, 2008), the effect of reverse induction on volatile induction still warrants investigation.

Timing of attack. Induction of the signal-transduction pathways occurs with a specific temporal pattern, and so a second attacker will have a certain timeframe during which it can have the greatest impact on the defense signaling network and subsequent volatile induction. Of particular interest is the case of hemi-biotrophic pathogens, which are biotrophs in the initial stages of infection, and then adopt a necrotrophic lifestyle, each theoretically inducing different signal-transduction pathways in the plant. Knowing this, how is crosstalk and volatile induction affected if an herbivore subsequently arrives in either the biotrophic or nectrotrophic phase?

Abiotic conditions. Changes in environmental conditions hold great potential to alter induced volatile emissions, both in terms of composition and quantity. Factors such as light, temperature, humidity, soil moisture, nutrient availability and ozone can all affect HIPV emission (Gouinguené & Turlings, 2002; Holopainen & Gershenzon, 2010; Loreto & Schnitzler, 2010). Such factors should be considered as they can have significant effects on volatile blend characteristics.

Other phytohormones. Recent research in plant-pathogen interactions indicates that several other phytohormones play a larger role in modulating plant defense than previously thought. Among many examples, auxin appears to disrupt biosynthesis of SA and to modify JA signaling, abscisic acid can antagonize JA-ET signaling (Pineda *et al.*, 2012), and brassinosteriods can lead to increased plant resistance towards biotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). It is clear that research on subcellular mechanisms of plant defense should look further than the classic trio of SA, JA and ET, and the impact other phytohormones may have on volatile induction is not yet known.

A more holistic approach is needed in the study of the ecology of induced plant volatiles during multiple attack, by integrating research approaches from the molecular to the ecological level, in order to gain a more comprehensive view of the mechanisms involved throughout the different levels of biological organization. More specifically, issues to address are how multiple attack affects the signal-transduction network, and how these changes then affect biosynthesis of volatiles compounds, as well as their emission patterns. And finally, how does variation in emission patterns affect the members of the third trophic level that depend on VOCs for e.g. host or prey location. However most of the knowledge on the mechanisms was gained from the model plant *Arabidopsis thaliana*. There is a need to use current knowledge gained from this system as a starting point for investigating ecologically relevant model systems. Moreover, research needs to be conducted out in the field as well as in the laboratory, as VOC emission patterns obtained under laboratory conditions may be different from patterns observed when a study system is in a natural environment, where various biotic and abiotic factors can have a strong influence on the emission of compounds (Kigathi *et al.*, 2009). Only by combining both approaches can we have a better understanding of the ecological functions of plant volatiles.

Plants are members of complex communities. Initial attack by one of the community members may have long term effects on community dynamics (Van Zandt & Agrawal, 2004; Poelman *et al.*, 2008a; Poelman *et al.*, 2010). In order to understand such community dynamics and the underlying processes that shape them, it is important to understand

how community members modify the expressed plant phenotype. An important component of the plant phenotype consists of its emission of VOCs as the VOC blend provides phenotypic information at a distance of the plant. This VOC-aspect of the plant phenotype mediates interactions with various community members (Dicke & Baldwin, 2010). So far, induced plant volatiles have been especially investigated in the context of plant-arthropod interactions. However, first evidence shows that plant pathogens can have important impacts on induced plant volatiles as well and, therefore, integrating plant-arthropod and plant-pathogen interactions into studies on the effects of HIPV on community dynamics of plants and their attackers and members of higher trophic levels will be an important next step. This integrative approach is likely to unravel new insights into the community ecology of plant-based communities.

Acknowledgements

The authors thank Arjen Biere and Alison Bennett for their constructive comments on an earlier version of the manuscript. This work, as part of the European Science Foundation EUROCORES Programme EuroVOL was supported from funds by the Earth and Life Sciences Council of the Netherlands Organisation for Scientific Research.

Chapter 3

Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore

Camille Ponzio, Rieta Gols, Berhane T. Weldegergis & Marcel Dicke

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

Published in a modified form in Plant, Cell and Environment, 37, 1924-1935 (**2014**)

ABSTRACT

Plants respond to herbivory with the emission of plant volatiles, which can be used by the herbivores' natural enemies to locate their hosts or prey. In nature, plants are often simultaneously confronted with insect herbivores and phytopathogens, potentially interfering with the attraction of the herbivores' enemies as a result of modifications of the induced volatile blend. Here we investigated parasitoid (*Cotesia glomerata*) attraction to volatiles of plants challenged by different attackers, either alone or in combination with *Pieris brassicae* caterpillars, hosts of *C. glomerata*. We used a natural system consisting of *Brassica nigra* plants, eggs and larvae of *P. brassicae*, *Brevicoryne brassicae* aphids and the bacterial phytopathogen *Xanthomonas campestris* pv. *campestris*. In all cases, parasitoids successfully located host-infested plants, and wasp foraging behavior was unaffected by the simultaneous presence of a non-host attacker or host eggs. Analysis of the volatile emissions showed that the volatile blends of caterpillar-infested treatments were different from those without caterpillars. Furthermore, dually attacked plants could not be separated from those with only caterpillars, regardless of non-host identity, supporting the behavioral data. Our results suggest that, in this system, indirect plant defenses may be more resistant to interference than is generally assumed, with volatiles induced during dual attack remaining reliable indicators of host presence for parasitoids.

Keywords: *Pieris brassicae*, *Cotesia glomerata*, tritrophic interactions, HIPV, indirect defense

Introduction

It is well known that insect herbivory changes the emission of plant volatiles, which are important cues in the foraging behavior of the natural enemies of these herbivores. These volatiles play key ecological roles in structuring associated insect communities as they do not only influence the behavior of the natural enemies but also that of the herbivores and even insects at the fourth trophic level (Vet & Dicke, 1992; Dicke, 1999; Turlings & Wackers, 2004; Dicke & Baldwin, 2010; Fatouros *et al.*, 2012; Poelman *et al.*, 2012). Most studies demonstrating the attraction of natural enemies to herbivore-induced plant volatiles (HIPV) have done this when the plant is attacked by single herbivore species. However, in nature, complex tritrophic interactions with simultaneous or sequential attack is the norm. Interplay between attackers can have strong implications for the interaction between plants and natural enemies of their associated herbivores, via modifications of the emitted volatile blend (Dicke *et al.*, 2009; Ponzio *et al.*, 2013).

While the direct effects of multiple attack on herbivore life-history parameters and behavior have been investigated in detail (For reviews see Denno *et al.*, 1995; Kaplan & Denno, 2007; Stam *et al.*, 2014), to date comparatively fewer studies have investigated HIPVs and the ensuing effects on tritrophic interactions in the context of multiple herbivore attack (Shiojiri *et al.*, 2001; Rodriguez-Saona *et al.*, 2003; Zhang *et al.*, 2009; Erb *et al.*, 2010; Hare & Sun, 2011). Insects are not the sole organisms to attack plants; plant pathogens also commonly infect plants, yet little attention has been paid to the effects of plant pathogen infection on tritrophic interactions despite the strong prevalence of pathogens in natural systems, frequently co-occurring with herbivory (Tack & Dicke, 2013). Until recently, research on plant-insect and plant-pathogen interactions have developed largely independently from one another, though plant defense against both is regulated by the same general mechanisms (Pieterse & Dicke, 2007; Ballhorn, 2011; Pieterse *et al.*, 2012). Interestingly, not only are pathogens capable of inducing volatile emissions in plants (Croft *et al.*, 1993; Doughty *et al.*, 1996; Huang *et al.*, 2003), some also induce compounds which are often associated with herbivory, such as the green leaf volatiles (*Z*)-3-hexenol and (*E*)-2-hexenal (Croft *et al.*, 1993). It appears that as with multiple insect herbivory, concomitant challenge by a pathogen and herbivore can affect HIPV composition and emission rates (Ponzio *et al.*, 2013). In maize, for instance, plants simultaneously challenged by the fungus *Setosphaeria turcica* and *Spodoptera littoralis* caterpillars had total volatile emission rates nearly 50% lower than with herbivory alone, yet the host searching behavior of two parasitic wasp species was not affected (Rostás *et al.*, 2006). Meanwhile, in peanut plants, dual infestation with a pathogen and a herbivore

led to enhanced wasp attraction (Cardoza *et al.*, 2003b). It is apparent that the potential effects of pathogen infection on HIPV emissions have long been underestimated, and warrant further attention.

At the molecular level, defense against different attacking organisms is modulated by the activation of different signaling pathways. It is generally accepted that phloem-feeding herbivores and biotrophic pathogens primarily induce the salicylic acid (SA) signaling pathway, while leaf-chewing herbivores and necrotrophic pathogens usually trigger the jasmonic acid (JA) and ethylene (ET) signaling pathways (Glazebrook, 2005; Pieterse & Dicke, 2007; Pieterse *et al.*, 2012). These different phytohormones have been shown to also be involved in the induction of plant volatiles (van Poecke & Dicke, 2004). However, crosstalk can occur between these pathways, where induction of one pathway can have positive or negative regulatory effects on other pathways, and this is particularly the case between the SA and JA pathways (Koornneef & Pieterse, 2008; Spoel & Dong, 2008; Pieterse *et al.*, 2012; Thaler *et al.*, 2012). Multiple attack may lead to quantitative and/or qualitative changes in the emitted volatile blend compared to the single attack situation, particularly when the challengers induce opposing signaling pathways. Consequently, herbivore finding by carnivorous natural enemies may be compromised if multiple attack leads to significant alterations of the plant volatile blend characteristics induced by their herbivorous victim. As a result the blend may no longer be a reliable indicator of host presence, though still little is known about the specificity of individual compounds or mixtures of compounds that are important in foraging behavior of natural enemies (Gols *et al.*, 2011). The importance of specific compounds was clearly demonstrated in Lima bean plants, where simultaneous attack by spider mites (*Tetranychus urticae*) and whiteflies (*Bemisia tabaci*) reduced emissions of only a few compounds, especially (*E*)-β-ocimene, compared to plants with only spider mites. As a result, the attraction of the spider-mite predator *Phytoseiulus persimilis* was negatively affected, which could be restored by complementing the blend with (*E*)-β-ocimene. (Zhang *et al.*, 2009).

The aim of the present study was to investigate the attraction of a parasitoid to plant volatiles induced by its natural herbivorous host as well as other plant attackers which are known to activate different signaling pathways. To date, studies investigating parasitoid foraging under multiple attack scenarios have largely done so using only one non-host attacker. Here we investigated the effects of several non-host attackers using a natural system consisting of the wild annual crucifer, *Brassica nigra* L. (Brassicaceae) and some of its naturally associated attackers: eggs and larvae of the large cabbage white butterfly (*Pieris brassicae* L. Lepidoptera, Pieridae), the cabbage aphid (*Brevicoryne brassicae* L., Hemiptera, Aphididae) and the necrotrophic bacterial phytopathogen, *Xanthomonas*

campestris pv. *campestris* (hereafter referred to as *X. campestris*). These attackers were selected based on the defense signaling pathways they induce. While plant defenses against leaf-chewing caterpillars are induced via the JA-signaling pathway, eggs and aphids are known to primarily induce the SA-signaling pathway (Bruessow *et al.*, 2010; Giordanengo *et al.*, 2010), and the *Xanthomonas* pathogen is thought to induce defenses via all three of the primary pathways (Ton *et al.*, 2002). Different attackers were introduced on the plant under single and dual attack scenarios, with the latter scenarios focused more specifically on the systemic effects of the initial inducer. The focal interaction studied here is that between the larval endoparasitoid wasp *Cotesia glomerata* L. (Hymenoptera, Braconidae) and its preferred host in the Netherlands, early developmental stages of *P. brassicae* caterpillars.

The effects on the attraction of naive *C. glomerata* to plant volatiles under various attack scenarios was investigated and the volatiles emitted by the plants were collected and identified to reveal the underlying chemical basis of the effects on wasp behavior. Due to the inherently different nature of the three initial attackers, we expected them to lead to significant effects on the volatile profiles emitted by the infested plants, as well as on the degree of parasitoid attraction.

Materials and Methods

Plant and insect material

Seeds from black mustard plants (*B. nigra*) were collected from a local population growing along the Rhine river in Wageningen (the Netherlands). Plants were grown in a greenhouse at 22 ± 2 °C, 60-70% r.h., 16:8 light:dark regime. Four to five week old plants were used for all experiments. The herbivores, *P. brassicae* and *B. brassicae,* were reared on Brussels sprout plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in a climate-controlled room or greenhouse at 22 ± 2 °C, r.h 60-70% and a photoperiod of L:D of 16:8 h. The parasitoid, *C. glomerata,* was reared on *P. brassicae* under similar conditions. Fresh cocoons of *C. glomerata* were placed in a 30 x 30 x 30 cm cage (Bugdorm, Taiwan) supplied with a 6% sucrose solution and honey. The cage with wasps was kept in a climate-controlled cabinet at 21 ± 1°C and a light-dark regime of 16:8 h. Adults were allowed to mate and were 3 to 7 days old when used in the wind tunnel bioassays (see below). Only adult females were used and they had no previous experience with hosts, host products or plants (i.e. they are considered naïve). Individuals were only used once.

Plant treatments

Plants were incubated under similar conditions as the insect rearing in a climate-controlled environment and were maintained in 35 x 35 x 60 cm mesh cages (Vermandel, the Netherlands), one treatment per cage. For treatments involving *X. campestris,* as well as for their associated pathogen-free treatments, the cages were covered with a clear plastic sheet for the first 24 h in order to insure sufficiently high humidity (minimum 80% RH) for optimal pathogen development. The following 10 different treatments were used:

1) healthy uninfested control plants (C),
2) plants infested with 20 newly hatched *P. brassicae* larvae (P),
3) plants infested with 50 *B. brassicae* aphid nymphs (A50),
4) plants infested with 100 *B. brassicae* aphid nymphs (A100),
5) plants infested with 50 *P. brassicae* eggs (E),
6) plants inoculated with *X. campestris* (X),
7) plants infested with 50 *B. brassicae* aphid nymphs and 20 newly hatched *P. brassicae* (AP50),
8) plants infested with 100 *B. brassicae* aphid nymphs and 20 newly hatched *P. brassicae* (AP100),
9) plants infested with 50 *P. brassicae* eggs and 20 newly hatched *P. brassicae* (EP),
10) plants inoculated with *X. campestris* and 20 newly hatched *P. brassicae* (XP).

The induction of plant volatiles in response to eggs, aphids or pathogens generally takes longer than the induction of volatiles in response to caterpillar feeding (De Vos *et al.*, 2005; Bruessow *et al.*, 2010). Therefore, plants treated with eggs, aphids or the pathogen were incubated for 72h, whereas caterpillars were introduced for the final 24 h before testing in the wind tunnel (see below). The initial egg, aphid or pathogen challengers were applied on the youngest fully developed leaf, whereas *P. brassicae* were introduced onto the younger adjacent leaf in dual-infestation treatments or a leaf of similar age in treatments with only caterpillar damage. For the treatments including *P. brassicae* eggs, oviposition was contained to the youngest fully expanded leaf by covering the plant with a zippered mesh bag that allowed only the selected leaf to protrude. Plants were then placed in a cage containing over 100 *P. brassicae* adults, and were removed once 1-2 egg clutches (about 50 eggs) were laid. Any extra eggs were immediately removed with a fine brush. For treatments involving *B. brassicae* aphids, first and second instar nymphs were used, to insure that no reproduction would occur during the induction period.

All *X. campestris* cultures were started from an initial culture obtained from the Plant-

Microbe Interactions group of Utrecht University (the Netherlands) that was stored at -80°C in 50% glycerol. Inoculum was obtained by incubating 250 µl of the frozen stock in 50-ml erlenmeyer flasks containing 30 ml of Difco Nutrient Broth. The flask was sealed by a cotton wool plug and aluminum foil and then placed in a shaker at 28 °C, 180 rpm for 18 h. The broth was then transferred to 50-ml tubes, and centrifuged at 3000 *x g* for 10 min. The bacterial cells were resuspended in a 10 mM $MgSO_4$ buffer solution and adjusted to a final density of 10^7 CFU/ml by measuring the absorbance with a spectrophotometer set at 600 nm. Plants were inoculated by infiltrating a zone of circa 2 cm^2 with a needleless syringe, on the abaxial side of the youngest fully expanded leaf. Control and *P. brassicae* infested plants were infiltrated in a similar manner with buffer solution only.

For each of the 4 treatments (treatments 7-10) involving dual attack, plants were first induced in the same manner as the treatments with one attacker (as described above) and then 48 h after applying the first challenger, 20 freshly emerged first instar *P. brassicae* caterpillars were placed on the systemic leaf directly above, and allowed to feed for a further 24 h.

Wind tunnel assays

Dual choice tests were carried out in a wind tunnel as previously described by Geervliet, Vet & Dicke (1994), at 25 °C, 60% r.h. and with a wind speed of 10 cm s^{-1}. Each of the single and dual infestation treatments were tested against a control plant, with dually infested plants additionally being tested against a *P. brassicae*-infested plant. The plants were placed in the tunnel 30 minutes prior to the bioassay in order to allow the dissipation of any volatiles resulting from mechanical damage during handling. Thirty minutes before the bioassay, naïve female *C. glomerata* wasps were isolated in glass vials sealed with cotton wool. Just before release in the wind tunnel, the wasps were sensitized by offering them a Brussels sprout leaf on which *P. brassicae* caterpillars had previously fed, with the caterpillars and their products removed. This procedure enhances the females' motivation to forage without affecting odor preference (Geervliet *et al.*, 1994). Groups of 5 vials were placed on the release platform, 70 cm downwind from the two plants. Wasps were given 15 minutes to respond, i.e. land on a plant. Non-responding wasps were recorded, however they were excluded from the choice statistical analysis. In total, 10 to 20 wasps were released per plant replicate, in order to obtain a minimum of 7 responding wasps. Each wasp was only used once. The position of the plants was swapped each time a new group was released. A total of 10 replicates with newly

prepared plants were tested for each treatment pair. The bioassays were performed in blocks, with the identity of the first attacker (eggs, aphids or pathogen) as the block factor. Test combinations were randomized within each block.

Collection of volatiles and headspace analysis

Plant volatiles were collected in order to investigate if differences in volatile profiles could explain the observed parasitoid behavior. For each treatment, 10 replicates were sampled. All insects remained on the plants during volatile trapping. The volatiles of the plants involving inoculation with *X. campestris*, along with the associated control and *P. brassicae* damaged plants, were collected and analyzed separately at a later date. In order to prevent any contribution from the collection set-up to the plant volatile profile and to make necessary corrections, air from empty jars were sampled at regular intervals. Pots were wrapped using aluminum foil and plants were placed in 30 l glass jars 30 min before trapping commenced. Compressed air was filtered through activated charcoal before entering the glass jar with the plant and volatiles were collected by drawing air out of the jars via an external pump through a stainless steel cartridge filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) at a rate of 300 ml min^{-1} for 2 h. The aerial portion of each plant was weighed immediately after volatile trapping. The Tenax TA filled cartridges with the trapped headspace samples were dry-purged for 15 min under a nitrogen (N_2) flow at 50 ml min^{-1} and stored at ambient temperature.

Headspace samples were analyzed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific) quadruple mass spectrometer. Volatiles were desorbed from the sampling cartridges using a thermal desorption system (Ultra 50:50, Markes, Llantrisant, UK) at 250 °C for 10 min with a helium flow of 20 ml min^{-1}. Analytes were focused at 10 °C on a thermally cooled solvent trap (Unity, Markes) during the entire desorption time and then the temperature of the cold trap was raised through ballistic heating at 40 °C s^{-1} to 280 °C, which was then kept for 10 min, while the volatiles were transferred in splitless mode to a ZB-5MSi analytical column (30 m, 0.25 mm i.d., 1.0 μm film thickness, (Phenomenex, Torrence, CA, USA)). The GC was held at an initial temperature of 40 °C and was immediately raised at 5 °C min^{-1} to 280 °C and was held for 4 min under a constant column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 35–400 m/z with a scan rate of 4.70 scans s^{-1}. MS transfer line and ion source were set at 275 and 250 °C, respectively. Tentative identifications of compounds were made by comparison of mass

spectra with the mass spectral databases in libraries of NIST 2005 and the Wageningen Mass Spectral Libraries Database 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, divided by the fresh plant weight, were further used in the statistical analysis.

Statistical analysis

To investigate whether parasitoid preferences and response rates differed when various combinations of plant treatments were offered, the data were analyzed using logistic regression in SAS version 9.2 with plant treatment as a fixed factor. In case of overdispersion, we corrected for this by allowing the variance functions of the binomial distribution to have a multiplicative overdispersion factor. In the comparison with control plants, the number of wasps choosing the attacker-infested plants out of the total number of responding wasps was entered as the response variable. In the analysis of dual versus single attack, the number of wasps choosing the dually infested plant out of the total number of responding wasps was entered as the response variable. A similar approach was used to determine differences in overall response rates. Each bioassay with one set of plants served as a replicate. To determine within each comparison whether there was a significant preference for one of the offered plant treatments, we tested H_0: logit=0.

The volatile emission patterns, quantified as peak areas divided by the fresh mass of the plant, were analyzed through multivariate data analysis using PSL-DA (projection to latent structures discriminant analysis). This projection method determines if samples belonging to the different treatment groups can be separated on the basis of quantitative and qualitative differences in their volatile blends. To do this, a Y-data matrix of dummy variables are included, assigning a sample to its respective class. The PLS-DA extension in the SIMCA-P+ 12.0 software program (Umetrics AB, Umeå, Sweden) then approximates the point 'swarm' in X (matrix with volatile compounds) and Y in PLS components in such a way that the maximum covariation between the components in X and Y is achieved. The results of the analysis are visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components as well as the relationships among the variables. Data were log-transformed, mean-centered and scaled to unit variance before they were subjected to the analysis. Compounds were excluded if they were present in less than half of the samples in only one of the treatments.

Results

Host-infested plants remain attractive to wasps even in the presence of non-hosts

In the experiment with *P. brassicae* eggs as the first treatment, in all cases the wasps significantly preferred the volatiles from the egg-infested plant when offered against volatiles from a clean control plant though the strength of the preference differed between the treatments (GLM, χ^2=36.14, P <0.0001, Fig. 1a). Wasp preference for an infested plant was stronger if the caterpillar host was present, both in a single and dual attack situation, compared to plants with eggs alone. In the experimental block with aphids (Fig 1b), here too wasp preference significantly varied among treatments (GLM, χ^2=153.73, P <0.0001) and the behavioral choices were aphid density-dependent when offered against control plants. At a density of 50 aphids wasps did not discriminate between aphid-infested and control plants, while plants with 100 aphids were avoided by the wasps. However, when plants were dually infested with aphids and caterpillars the density-dependent effect disappeared, with wasps significantly preferring volatiles from plants infested with caterpillars plus 50 or 100 aphids over healthy control plants (Fig. 1b). The strength of the preference was similar to when they were offered a caterpillar-infested plant against a control plant. In the bioassays using *X. campestris*, all induced plants were attractive to the parasitoids when tested against control plants, though the strength of the preference depended on the treatment (GLM, χ^2=10.18, P =0.0062); the preference for plants induced with only the pathogen was weaker compared to when plants contained caterpillar hosts (Fig. 1c). To test for more subtle effects of the non-host attacker on parasitoid attraction to caterpillar-induced plant volatiles, dually challenged plants were also tested against plants with caterpillar hosts only (Fig. 1d). There were no significant differences between the different tested treatment combinations (GLM, χ^2=6.29, P =0.0981). However, wasps were significantly more attracted to volatiles from plants dually challenged by *X. campestris* and hosts, which was not the case for the other treatment combinations (with eggs or aphids as second attacker) where wasp preference was not different from a 50:50 distribution.

Despite the observed differences in preference, when response rates of the wasps were analyzed (i.e. the proportion of released wasps that landed on a plant), no effect of treatment was found between treatment pairs, with the exception of the experimental block using aphids as the initial attacker (GLM, χ^2=17.92, P =0.0013, Fig.1b). Here, infestation with aphids alone had an effect on the response rates. Plants infested with 50 aphids tested against healthy plants resulted in a response rate that was significantly lower than in any of the other treatment combinations that included caterpillars. However,

plants infested with 100 aphids and tested against healthy plants had wasp response rates that were not significantly different from any of the other treatments.

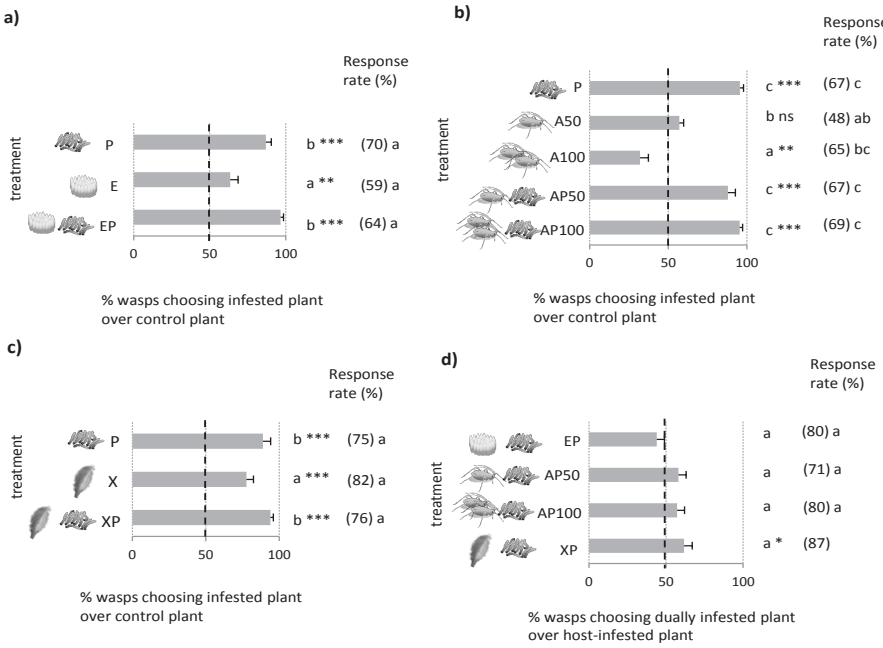


Figure 1. Choice of *C. glomerata* wasps in a two-choice setup in a wind tunnel (percentage (±SE, n=10) of wasps to treatment indicated on Y-axis) when the alternative odor source is a clean control plant (for **(a)**, **(b)** and **(c))** or a caterpillar infested plant **(d)**. Significant differences between treatments ($P < 0.05$) are indicated with different letters. Treated plants were challenged with: 20 *P. brassicae* caterpillars (P); 50 *P. brassicae* eggs (E); 50 (A50) or 100 (A100) *B. brevicoryne* aphids; *Xanthomonas* pathogen (X); caterpillars and *P. brassicae* eggs (EP); caterpillars with 50 (AP50) or 100 (AP100) aphids; caterpillars and pathogen (XP). Asterisks indicate a preference which is significantly different from a 50:50 distribution within a choice test : * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns is not significantly different.

Headspace analysis in the different attack scenarios

A total of 48 different volatile compounds were detected across all treatments in the headspace of the egg and aphid experiments (Table 1), and 45 compounds in the experiment with *X. campestris* as the initial attacker (Table 2). Overall, all plants emit the same compounds, but in different proportions. Thus, the composition of the blend varies according to the treatment.

Table 1. Volatile emissions[a] by *Brassica nigra* plants from uninfested plants (C) and in response to *Pieris brassicae* eggs (E), 50 *Brevicoryne brassicae* aphids (A50), 100 aphids (A100), *P. brassicae* caterpillars (P), eggs and caterpillars (EP), 50 aphids and caterpillars (AP50), 100 aphids and caterpillars (AP100)

ID[b]	Treatment→ / Compound ↓	C (N=10)	E (N=10)	A50 (N=9)	A100 (N=10)	P (N=10)	EP (N=10)	AP50 (N=10)	AP100 (N=10)
Ketones									
1	2-Butanone	70.3 ±4.6	76.7 ±8.3	78.1 ±8.1	69.4 ±5.9	65.7 ±6.6	75.7 ±7.2	79.7 ±7.8	67.6 ±6.8
4	3-Methyl-2-butanone	22.4 ±5.6	14.5 ±2.4	v16.8 ±2.4	16.7 ±3.4	26.3 ±4.5	26.4 ±5.1	27.4 ±5.7	17.2 ±3.2
6	3-Pentanone	35.5 ±4.8	18.6 ±3.0	29.3 ±5.3	41 ±17	23.4 ±4.1	22.9 ±2.9	40.56 ±11.0	40 ±17
9	3-Methyl-2-pentanone	6.2 ±3.0	6.8 ±1.8	2.68 ±0.30	3.16 ±0.60	4.9 ±0.9	5.6 ±1.2	9.1 ±3.6	3.91 ±0.70
Alcohols									
5	1-Penten-3-ol	147 ±38	70.67 ±13.0	187 ±60	125 ±30	100 ±16	114 ±24	112 ±22	113 ±40
11	(Z)-3-Hexen-1-ol	54 ±18	33.5 ±9.7	99 ±45	57 ±19	57.4 ±8.8	132 ±71	81 ±24	76 ±30
20	Dihydromyrcenol	140 ±70	115 ±71	47.1 ±14.3[8]	94 ±50[9]	130 ±87	64 ±33	29.9 ±7.1	60 ±17
28	1-Decanol	39.8 ±3.6	38.8 ±4.0	28.6 ±4.6	39.4 ±5.1	34.5 ±3.5	45.4 ±5.5	34.8 ±3.4	38.2 ±6.4
42	1-Dodecanol	123.2 ±7.2	120 ±16	105 ±17	112 ±16	111.8 ±12.6	109.3 ±11.0	115.1 ±14.5	113.9 ±14.6
Esters									
16	(Z)-3-Hexen-1-ol, acetate	319 ±125	124 ±39	312 ±148	245 ±79	231 ±36	366 ±142	209 ±49	368 ±155
29	cis-2-tert-Butylcyclohexyl acetate	9.16 ±1.40	9.4 ±2.4[9]	6.0 ±1.0	6.7 ±1.0	11.0 ±2.6	9.6 ±3.4[9]	7.8 ±2.2	6.65 ±1.1
N and/or S containing compounds									
2	2-Butenenitrile	3.81 ±0.70	2.94 ±0.90[9]	8.3 ±2.2	10.4 ±3.6	18.8 ±3.6	16.5 ±3.4	34.3 ±8.4	21.7 ±5.9
3	3-Butenenitrile	23.1 ±5.6	16.0 ±3.5	30.1 ±9.7	75.6 ±35	136 ±45	81.6 ±15.2	186 ±51	130 ±36
7	2-Methylbutanenitrile	14.7 ±2.6	9.4 ±1.0	15.6 ±2.7	25.6 ±9.1	43.8 ±14.9	37.8 ±10.4	41 ±19	47.2 ±13.1
8	Dimethyl disulfide	22.1 ±6.1	32.6 ±11.3	56 ±35[8]	27.7 ±4.8	20.1 ±4.9	43.2 ±13.2	42.3 ±10.5	61 ±44
12	Allyl Isothiocyanate	289 ±107[9]	90 ±24[9]	258 ±86[8]	169 ±52	398 ±86	371 ±99	590 ±224	207 ±33

ID[b]	Treatment→ / Compound ↓	C (N=10)	E (N=10)	A50 (N=9)	A100 (N=10)	P (N=10)	EP (N=10)	AP50 (N=10)	AP100 (N=10)
Aldehydes									
10	(*E*)-2-Hexenal	9.06 ±1.30	7.67 ±0.50	9.4 ±1.7	8.7 ±1.7	10.0 ±1.3	9.38 ±1.40	7.66 ±1.10	7.51 ±0.90
13	(*E*)-4-Oxo-2-hexenal	51 ±21 [9]	18.4 ±4.5 [8]	62 ±34 [8]	44 ±16 [9]	51.2 ±11.3	46.7 ±10.8	42 ±16	39.9 ±11.8
Terpenoids									
14	Myrcene	15.6 ±2.7	13.0 ±3.5	8.0 ±0.9	10.12 ±1.30	12.2 ±1.6	10.3 ±1.9	7.9 ±1.0	10.06 ±1.90
17	2-Carene	2.59 ±0.70 [8]	2.43 ±0.40 [9]	2.6 ±0.8 [8]	2.23 ±0.50 [8]	2.33 ±0.50 [9]	2.6 ±0.50 [8]	2.31 ±0.60 [9]	1.97 ±0.50 [8]
18	β-Phellandrene	19.7 ±4.2	12.63 ±1.20	15.1 ±5.0	13.1 ±3.2	13.5 ±3.4	16.9 ±3.1	13.5 ±3.0	11.60 ±2.7 [9]
19	1,8-Cineole	2.65 ±0.50	4.09 ±0.70 [9]	2.98 ±0.70 [8]	2.67 ±0.70 [8]	4.39 ±0.60	3.28 ±0.80 [9]	3.4 ±0.60	3.2 ±0.60 [9]
21	γ-Terpinene	2.71 ±0.30	2.63 ±0.50	1.69 ±0.30 [8]	1.68 ±0.3 [9]	2.41 ±0.40	2.49 ±0.6	2.9 ±1.0	2.32 ±0.50
22	(*E*)-DMNT[c]	2.5 ±2.5	2.4 ±2.4 [1]	4.14 ±2.7 [2]	2.8 ±2.8 [1]	136 ±38	205 ±67	100 ±20	104 ±20
23	Menthone	8.39 ±1.10	6.57 ±0.70	8.05 ±1.7	7.7 ±0.9	10.8 ±2.0	7.63 ±1.40	7.93 ±1.20	8.82 ±1.10
24	Isomenthone	2.39 ±0.30	1.89 ±0.30 [9]	2.58 ±0.6	2.22 ±0.40 [9]	3.42 ±0.60	2.49 ±0.40	2.24 ±0.60 [7]	2.91 ±0.50 [9]
25	Menthol	44.3 ±5.8	34.9 ±3.1	42.15 ±7.7	42.7 ±5.1	69.1 ±20.1	41.9 ±7.7	46.6 ±9.0	52.8 ±8.6
31	7-α-H-Silphiperfol-5-ene	193 ±55	118 ±30	123.36 ±39.6	88.7 ±12.2	148.42±55.1 [7]	66.1 ±13.7 [9]	132 ±56 [9]	187 ±69 [9]
32	Presilphiperfol-7-ene	35 ±17	16.7 ±5.5	22.03 ±7.4 [8]	20.1 ±5.0	11.1 ±3.3 [7]	10.0 ±3.2 [9]	18.8 ±6.4 [9]	20.8 ±9.2 [9]
34	7-β-H-Silphiperfol-5-ene	73 ±21	46.9 ±12.0 [9]	44.33 ±12.9 [8]	34.3 ±5.6	64.4 ±26 [7]	26.1 ±5.5 [9]	52.7 ±22 [8]	80 ±31 [9]
35	Asterisca-3(15),6-diene	3.95 ±0.60	2.84 ±0.50 [9]	3.6 ±0.6 [8]	3.10 ±0.70 [9]	4.90 ±1.10	2.59 ±0.40	3.51 ±0.80	3.7 ±1.0 [9]
36	Silphiperfol-6-ene	32.7 ±9.4	22.3 ±5.9 [9]	19.98 ±6.6 [8]	15.7 ±2.7	32.1 ±13.7 [7]	11.1 ±2.4 [9]	23.1 ±9.2 [9]	39 ±16 [9]
37	Longicyclene	16.0 ±4.5 [2]	18.1 ±5.6 [9]	15.89 ±5.5 [8]	18.8 ±5.9	17.2 ±5.7	20.8 ±7.2 [9]	17.8 ±5.3	20.8 ±9.5
38	7-epi-α-Cedrene	21.4 ±6.7 [9]	20.6 ±6.9 [9]	6.51 ±2.2 [8]	5.08 ±1.30 [7]	8.2 ±2.5 [8]	7.3 ±2.1	8.9 ±2.6 [8]	7.9 ±3.0 [9]
40	α-Barbatene	10.5 ±2.0	14.5 ±5.0	12.64 ±2.2	5.17 ±1.10 [8]	16.7 ±5.5	11.1 ±3.9	8.0 ±1.5	9.8 ±2.8
41	β-Caryophyllene	28.9 ±9.6	14.0 ±10.2 [7]	61.2 ±50.2	15.3 ±10.2	15.01 ±5.3 [9]	46 ±35 [8]	9.5 ±3.6 [9]	16.7 ±7.3 [9]
43	α-Humulene	22.1 ±8.8 [8]	15.2 ±6.4 [8]	35 ±26.7 [9]	11.8 ±5.0 [9]	8.5 ±3.6 [6]	26 ±18 [8]	8.8 ±3.6 [8]	19.7 ±9.0

ID[b]	Treatment→ / Compound ↓	C (N=10)	E (N=10)	A50 (N=9)	A100 (N=10)	P (N=10)	EP (N=10)	AP50 (N=10)	AP100 (N=10)
44	(*E,E*)-alpha-Farnesene	1.12 ±1.10 [1]	ND	ND	6.1 ±2.5 [4]	17.9 ±7.6 [6]	8.0 ±3.8 [7]	13.6 ±2.4 [9]	5.2 ±2.4 [4]
45	α-Amorphene	5.06 ±0.80 [9]	4.41 ±0.70 [9]	4.75 ±1 [9]	4.26 ±0.50 [9]	4.65 ±0.40	4.48 ±0.5	3.08 ±0.80 [7]	4.28 ±0.70 [9]
46	(*E,E*)-TMTT[d]	3.9 ±2.6 [2]	4.3 ±4.3 [1]	2.59 ±2.6 [1]	ND	11.9 ±3.1 [7]	12.8 ±6.9 [5]	20.0 ±7.8 [8]	15.3 ±4.4 [7]
47	Unknown sesquiterpene	56.3 ±13.2	45.8 ±12.4	34.54 ±11.9	39.2 ±9.4 [9]	61 ±17	37.5 ±10.2 [8]	38.7 ±5.2 [8]	39.8 ±7.9 [9]
48	Unknown sesquiterpene	3.8 ±2.1 [3]	6.5 ±2.5 [5]	5.41 ±2.4 [4]	8.5 ±3.2 [5]	5.7 ±2.5 [6]	7.2 ±4.7 [3]	7.6 ±3.6 [6]	9.0 ±2.9 [6]
Unknown									
15	Unknown compound	51.8 ±10.3	55.6 ±12.5	52.77 ±10.3	50.0 ±10.5	54.5 ±14.0 [9]	60 ±19	62 ±17	49.9 ±12.5
26	Unknown compound	29.8 ±3.0	46.4 ±16	33.17 ±7.8	33.6 ±7.9	32.5 ±2.9	26.5 ±2.6	25.1 ±3.4	30.6 ±4.5
27	Unknown compound	32.3 ±2.2	52 ±19	36.57 ±9.1	37.9 ±8.9	32.5 ±2.9	29.8 ±3.1	27.2 ±3.6	33.4 ±4.7
30	Unknown compound	74.9 ±7.5	66.9 ±7.5	54.69 ±8.2	61.1 ±7.2	61.9 ±5.3	61.3 ±6.5	61.1 ±5.8	59.0 ±6.6
33	Unknown compound	18.8 ±3.8	19.4 ±5.2	17.77 ±4.4	16.6 ±4.8	15.1 ±3.1	18.6 ±4.2	22.0 ±5.7	14.4 ±3.6
39	Unknown compound	32.0 ±7.4	52 ±20	82.51 ±50	24.5 ±9.3	57 ±26	31.2 ±10.7	32.2 ±9.7	21.0 ±5.2
49	total	2226.36 ±186.5	1527.21 ±171.2	2107.07 ±385.8	1754.73 ±183.3	2407.3 ±348.1	2394.54 ±400.8	2473.21 ±411.5	2303.52 ±381.2

[a] Volatile emissions are given as mean peak area ±SE/g fresh weight of foliage divided by 10^4 with the number of samples between brackets

[b] ID corresponds with the numbers presented in Fig. 2b and 3b.

[c] (*E*)-DMNT= (E)-4,8-Dimethylnona-1,3,7-triene

[d] (*E,E*)-TMTT= 4,8,12-Trimethyl-1,3,7,11-tridecatetraene

Numbers in superscript following emission quantities give the number of samples in which the compound was detected, if it was not found in all samples

Table 2. Volatile emissions[a] by *Brassica nigra* plants from uninfested plants (C) and in response to *Xanthomonas campestris* pv. *campestris* (X), *Pieris brassicae* caterpillar feeding (P), *X .campestris* and caterpillars (XP)

ID [b]	Treatment→ / Compound ↓	C (N=11)	X (N=8)	P (N=11)	XP (N=10)
Ketones					
1	2-Butanone	11.0 ±3.2 [7]	17.9 ±6.0 [5]	17.5 ±4.3 [7]	15.1 ±4.8 [6]
4	3-Methyl-2-butanone	7.2 ±1.8	12.4 ±3.6	15.2 ±3.1	18.6 ±4.8
6	3-Pentanone	6.59 ±0.70	11.7 ±1.5	10.2 ±1.8	16.9 ±2.6
9	3-Methyl-2-pentanone	0.09 ±0.10 [2]	1.86 ±0.80 [5]	0.1 ±0.1 [1]	1.7 ±1.0 [4]
Alcohols					
5	1-Penten-3-ol	39.1 ±4.4	102.5 ±14.9	88±33	149 ±41
11	(*Z*)-3-Hexen-1-ol	9.1 ±2.1	38.4 ±12.9	62 ±45	77 ±30
27	1-Decanol	9.1 ±2.1 [10]	10.1 ±2.9 [6]	18.1 ±3.7	11.7 ±1.5
39	1-Dodecanol	36.1 ±2.9	52.2 ±9.5	42.7 ±4.6	5 ±4.5
Esters					
15	Hexyl acetate	25.0 ±2.2	37.6 ±6.9	27.2 ±2.2	32.2 ±3.5
16	(*Z*)-3-Hexen-1-ol, acetate	21.0 ±3.9	27.2 ±9.9	38.3 ±8.4	68 ±23
28	cis-2-tert-Butylcyclohexyl acetate	5.0 ±1.1	8.44 ±1.30	6.44 ±0.90	6.70 ±1.10
N and/or S containing compounds					
2	2-Butenenitrile	ND	1.7 ±0.4 [7]	6.3 ±1.8	8.7 ±2.9
3	3-Butenenitrile	1.39 ±0.90 [2]	9.1 ±1.0	39.6 ±13.9	52 ±20
7	2-Methylbutanenitrile	12.7 ±4.9	20.6 ±13.9	15.5 ±5.4	47 ±28
8	Dimethyl disulfide	8.25 ±1.20	18.1 ±2.8	10.6 ±2.7	15.5 ±5.3
12	Allyl isothiocyanate	85 ±21 [10]	284 ±87	414±143	283 ±42
Aldehydes					
10	(*E*)-2-Hexenal	1.31 ±0.20	2.99 ±1.40	4.3 ±2.3	3.37 ±0.80
13	(*E*)-4-Oxo-2-hexenal	8.6 ±2.3	11.4 ±4.1 [7]	23.2 ±12.6	18.1 ±6.9
19	Dihydromyrcenol	21.0 ±5.9	21.0 ±3.6	29.9 ±5.4	17.1 ±3.9
Terpenoids					
14	Myrcene	8.1 ±1.4	15.4 ±3.7	8.70 ±1.10	8.8 ±1.6
17	β-Phellandrene	3.25 ±0.80	7.1 ±2.5 [7]	3.20 ±0.70	4.12 ±1.10
18	1,8-Cineole	1.11 ±0.30	1.64 ±0.50 [7]	1.22 ±0.20 [10]	1.04 ±0.30 [8]
20	γ-Terpinene	1.04 ±0.20 [9]	2.41 ±1.10 [7]	1.14 ±0.20 [10]	1.35 ±0.40 [8]
21	(*E*)-DMNT [c]	ND	1.7 ±1.7 [1]	73 ±32	54.4 ±9.7
22	Menthone	2.4 ±0.4	2.91 ±0.7	2.5 ±0.3	2.86 ±0.70
23	Isomenthone	0.57 ±0.20 [8]	0.59 ±0.20 [5]	0.63 ±0.20 [9]	0.92 ±0.30 [7]
24	Menthol	11.7 ±1.8	13.8 ±2.1	13.38 ±1.40	15.6 ±3.5
30	7-α-H-Silphiperfol-5-ene	84 ±25 [10]	50 ±15	66 ±18	73 ±26
31	Presilphiperfol-7-ene	7.2 ±3.2 [8]	3.24 ±1.10 [7]	4.8 ±1.0 [10]	5.9 ±2.2
33	7-β-H-Silphiperfol-5-ene	19.6 ±5.9 [8]	10.9 ±3.5	17.8 ±6.0 [10]	20.9 ±8.3 [9]
34	Asterisca-3(15),6-diene	1.19 ±0.20	1.53 ±0.40	1.16 ±0.20	1.77 ±0.40

ID [b]	Treatment→ Compound ↓	C (N=11)	X (N=8)	P (N=11)	XP (N=10)
35	Silphiperfol-6-ene	15.0 ±4.7 [8]	4.84 ±1.30 [7]	12.2 ±4.8 [10]	13.5 ±5.2 [9]
36	7-epi-α-Cedrene	3.2 ±1.0 [7]	5.9 ±2.9 [5]	6.1 ±1.9 [9]	15.1 ±9.2 [7]
37	α-Barbatene	2.07 ±0.40 [10]	3.45 ±0.80 [7]	2.97 ±0.70	5.26 ±1.40
38	β-Caryophyllene	2.77 ±1.0 [10]	1.65 ±0.30	2.78 ±1.10	5.6 ±2.3
40	α-Humulene	4.5 ±1.7 [8]	0.56 ±0.20 [4]	2.49 ±1.20 [9]	3.3 ±1.0 [8]
41	(*E,E*)-α-Farnesene	3.9 ±1.8 [4]	ND	5.7 ±2.4 [6]	5.4 ±2.2 [5]
42	α-Amorphene	1.81 ±0.20	2.48 ±0.70	1.84 ±0.30 [10]	2.25 ±0.30
43	(*E,E*)-TMTT [d]	0.36 ±0.40 [1]	3.47 ±1.40 [4]	21.2 ±12.1 [8]	13.1 ±3.4
44	Unknown sesquiterpene	15.8 ±3.5	22.3 ±6.5	22.50±3.8	20.4 ±3.6
45	Unknown sesquiterpene	2.53 ±1.[10]	3.0 ±1.5	1.48 ±0.50	5.0 ±2.1
unknown					
25	Unknown compound	26.6 ±5.2	34.6 ±11.5	30.6 ±4.0	20.9 ±2.8
26	Unknown compound	25.4 ±4.9	32.9 ±10.6	29.5 ±4.0	21.0 ±2.8
29	Unknown compound	15.39 ±1.20	19.9 ±3.4	18.5 ±1.9	16.20 ±1.20
32	Unknown compound	2.01 ±0.10	2.99 ±0.50	2.34 ±0.20	2.86 ±0.30
46	total	557.84 ±44.3	920.92 ±120.9	1211.23 ±189.9	1206.44 ±249.9

[a] Volatile emissions are given as mean peak area ±SE/g fresh weight of foliage divided by 10^4 with the number of samples between brackets.

[b] ID corresponds with the numbers presented in Fig. 2b and 3b.

[c] (*E*)-DMNT= (*E*)-4,8-Dimethylnona-1,3,7-triene

[d] (*E,E*)-TMTT= 4,8,12-Trimethyl-1,3,7,11-tridecatetraene

Numbers in superscript following emission quantities give the number of samples in which the compound was detected, if it was not found in all samples.

A PLS-DA including the samples with both aphid densities, *P. brassicae* eggs, their associated dually infested treatments as well as control and *P. brassicae*-infested plants resulted in a model with one significant principal component (PC) (Fig. 2a). This PC separated the volatile blends of plants based on the presence or absence of caterpillars, irrespective of the presence of a second attacker. When additional PLS-DAs were conducted while excluding either the treatments containing eggs or the treatments having aphids, no additional significant PCs were found, despite the observed behavioral differences. Samples with different aphid densities could not be further separated. Additional pairwise comparisons of the samples treated with caterpillars

alone and two attacker species did not result in separation of the samples in any of the comparisons. Similarly, pairwise comparisons of samples collected from control plants and plants treated with eggs or aphids could not be separated based on the volatiles these plants emitted (data not shown.) Examination of the loading plot shows that a group of seven compounds contributed the most to explaining the variation in the model (Fig. 2b). These are *(E)*-4,8-dimethylnona-1,3,7-triene ((*E*)-DMNT), 2-butenenitrile, 3-butenenitrile, 2-methylbutanenitrile, *(E,E)*-α-farnesene, *(E,E)*-4,8,12-trimethyl-1,3,7,11-tridecatetraene ((*E,E*)-TMTT) and allyl isothiocyanate.

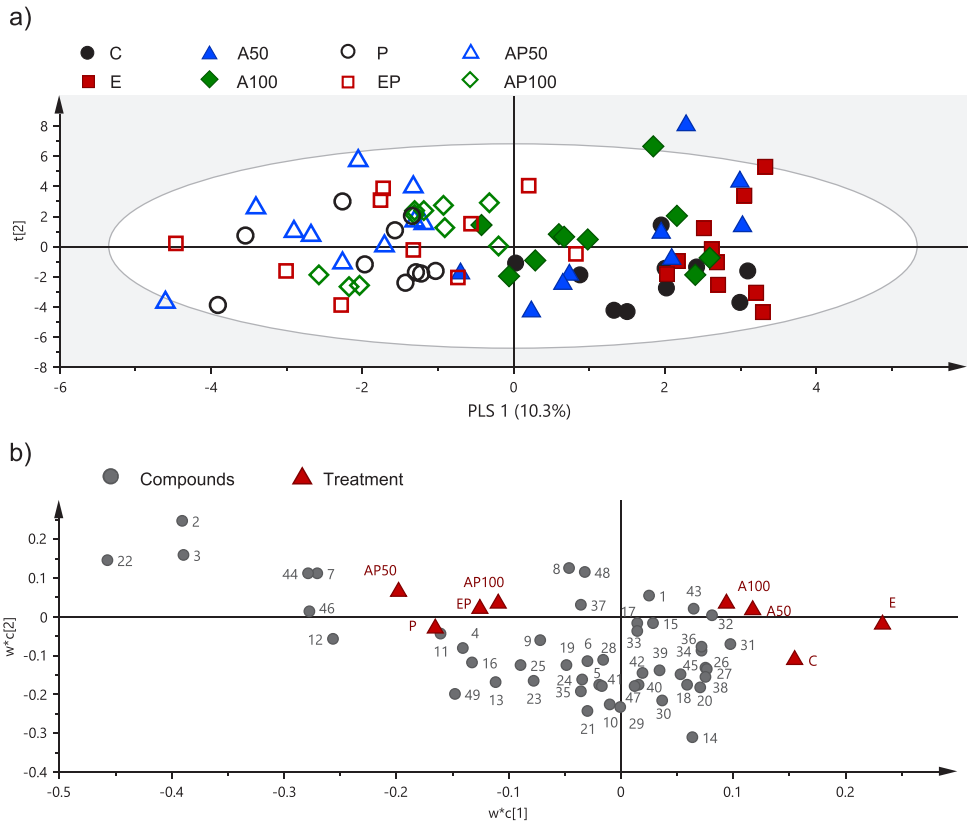


Figure 2. PLS-DA (Projection to Latent Structures Discriminant Analysis) comparison of the volatile compounds emitted by *B. nigra* plants sampled after 72 h. **(a)** Score plot of the samples, with the percentage of explained variation in parentheses. Plants were infested with *P. brassicae* eggs alone (E), eggs and *P. brassicae* caterpillars (EP), 50 *B. brassicae* aphids (A50), 50 aphids and caterpillars (AP50), 100 aphids (A100), 100 aphids and caterpillars (AP100), caterpillars (P) or were healthy control plants (C). The PLS-DA resulted in a model with one significant component: R^2X=0.103 R^2Y=0.11 Q^2=0.092 (the second axis is shown for representational purposes). The ellipse defines the Hotelling's T^2 confidence region, which is a multivariate generalization of the Students *t*-test and gives a 95% confidence interval for the observations. **(b)** Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 1.

When the samples from the pathogen experiment were analyzed, here also the resulting model had one significant PC, separating samples of control plants from those that were induced (Fig. 3a). Excluding the control samples resulted in a new significant PC, separating samples infected with only *X. campestris* from samples infested with *P. brassicae* caterpillars, both alone and in combination with *X. campestris* (Supporting Information Fig. S1). Pairwise comparison of caterpillar-infested and dually treated samples yielded no additional PCs though there is a clear tendency towards separation of the volatile profiles emitted by each treatment (data not shown) For this model, seven compounds most strongly contribute to explaining the differences between the treatments (Fig. 3b). These are *(E)*-DMNT, 2-butenenitrile, 3-butenenitrile, 1-penten-3-ol, *(E,E)*-TMTT, (*Z*)-3-hexen-1-ol, and 3-pentanone.

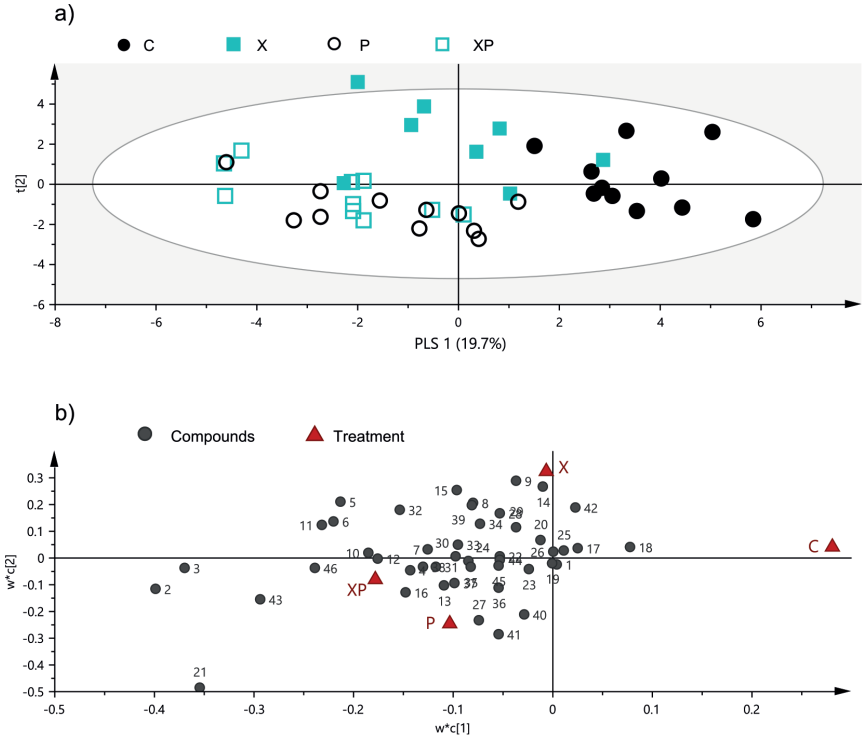


Figure 3. PLS-DA comparison of the volatile compounds emitted by individual *B. nigra* plants sampled after 72 h. **(a)** Score plot of the samples, with the percentage of explained variation in parentheses. Plants were infested with *P. brassicae* caterpillars (P), infected with *Xanthomonas* pathogen (X) pathogen and caterpillars (XP) or were healthy controls plants (C). The PLS-DA resulted in a model with one significant component: R^2X=0.198 R^2Y=0.239 Q^2=0.194 (the second axis is shown for representational purposes). The ellipse defines the Hotelling's T^2 confidence region (95%). **(b)** Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 2.

Discussion

In this study we investigated how the presence of a second, non-host, attacker may affect the attractiveness of the volatile blend for the parasitoid *C. glomerata*. The behavior of *C. glomerata* is well studied, particularly their ability to distinguish between volatiles from host-infested and clean plants, both on cultivated and wild plant populations (Mattiacci *et al.*, 1994; Mattiacci & Dicke, 1995; Geervliet *et al.*, 1996; Gols *et al.*, 2008; Gols *et al.*, 2009). We found that wasps responded to volatiles of host-infested plants, irrespective of the presence of a second attacker, and with the exception of plants dually challenged with *X. campestris*, they did not distinguish between dually infested plants and plants infested with only *P. brassicae* caterpillars. For the treatments involving *P. brassicae* eggs, such results were not unexpected, as egg-laden *B. nigra* plants are known to be attractive to *C. glomerata* (Fatouros *et al.*, 2012), though the wasps can only parasitize the early larval stages. Therefore, in this system, the simultaneous presence of egg and larval stages on a plant was not expected to negatively affect parasitoid foraging behavior. Conversely, aphid infestation, particularly at the higher density of 100 aphids, was expected to lead to interference with the volatiles induced by caterpillars on the basis of the opposing signaling pathways induced by phloem-feeding insects and caterpillars (Pieterse *et al.*, 2012; Zhang *et al.*, 2013). Contrary to the other two initial attackers -aphids or *P. brassicae* eggs- which are thought to primarily induce the salicylate signaling pathway, *Xanthomonas campestris* has been shown to induce a combination of salicylic acid, jasmonic acid and ethylene-dependent responses (Ton *et al.*, 2002). Therefore, the plant volatile response, as well as wasp behavior, were expected to be markedly different for the experiment including this attacker. Indeed, unlike for the other attacker combinations, plants challenged by both the bacteria and caterpillars were more attractive to the wasps than caterpillar-infested plants.

In the case of single infestation, the effects on wasp behavior were divergent. In the case of aphid infestation, the behavioral response was dependent on aphid density. Volatiles from plants infested with 50 aphids and from uninfested control plants were not discriminated, while volatiles from plants with 100 aphids were less attractive to the wasps than those from uninfested control plants. The higher response rate seen at the higher aphid density indicates that the wasps were actively seeking to avoid such plants. These density-dependent effects completely disappear once caterpillars are also involved. On the contrary, egg-infested and, remarkably, pathogen-infected plants proved to be attractive to wasps when presented against uninfested control plants. This attraction to *Xanthomonas*-infected plants may be due to the induction of the JA-

pathway by the pathogen, leading to the emission of compounds also emitted during caterpillar feeding. Only a few studies document the effects of pathogen infection on tritrophic interactions, and only one has investigated how natural enemies may perceive pathogen infection alone. Rostás et al. (2006) found that plants infected with only the necrotrophic fungus *Setosphaeria turcica* were not attractive to two parasitic wasp species, though co-infestation with the wasp's lepidopteran host restored their preference to levels comparable to those of plants which were infested with hosts only. This was despite the fact that dually challenged plants had total volatile emission rates 47% lower than plants with herbivores only, though the blends were qualitatively similar.

Chemical data supported to a large extent the outcome of the behavioral tests in those situations where hosts were present. Volatile profiles of all treatments with caterpillars were similar to each other, and separated from the volatile profiles of the caterpillar-free treatments. This supports the behavioral results, where wasps can reliably detect host-infested plants both in single and dual attack scenarios. The volatile profiles of dually and caterpillar-infested plants could not be segregated in the multivariate space, even at a finer level by comparing treatments pairwise. This effect was the same for all three initial attacker species used, despite them being taxonomically very dissimilar organisms. The introduction of host caterpillars overruled any effects on volatile emission that the first attacker alone may have had. In a study involving a different pathovar of *X. campestris* (*X. campestris* pv. *vesicatoria*), similar effects were found. Here, bell pepper (*Capsicum annuum*) plants infected with a compatible strain (i.e. exposure of plants to this strain results in successful infection) in combination with *Spodoptera exigua* caterpillar herbivory emitted a similar volatile profile to plants having only caterpillars (Cardoza & Tumlinson, 2006). As to dual infestation with aphids, in broad bean (*Vicia faba*), simultaneous pea aphid (*Acyrthosiphon pisum*) infestation suppressed emissions of some compounds normally induced by *S. exigua* caterpillars (Schwartzberg *et al.*, 2011). However, this latter study (Schwartzberg *et al.*, 2011) investigated effects on volatile emissions on a compound by compound basis, rather than assessing whole blend characteristics. Yet it is interesting to note that in the pairwise comparisons of the present study, the profiles of caterpillar-infested plants and plants challenged by *X. campestris* and caterpillars showed a marked tendency towards separation. There was likely no full separation because of the large amount of variation between samples, likely due to the use of a wild plant species in this study, which presents much more natural variation than in a homogeneous cultivar. This tendency towards separation is interesting, as in the behavioral assays it was also the only treatment combination where wasps showed a significant preference between singly and dually infested plants: in this case, preference for dually challenged plants.

Taking these observations together, along with the fact that plants infected with *X. campestris* alone are attractive to wasps indicates that *X. campestris* infection has an effect on the caterpillar-induced volatile blend. For the parasitoids, the attractiveness of *X. campestris*-induced volatiles may be problematic, as they would waste their time searching for caterpillar hosts on these plants.

With regards to plants damaged by only one of the attackers, we found no significant differences in volatile profiles between plants having different aphid densities, despite the divergent effects on wasp behavior. Though the multivariate data analysis did not segregate the volatile blends by treatment, wasps may be picking up on subtle changes in the blend characteristics that current GC-MS technology is not sensitive enough to detect, or alternatively, only the specific subset of volatiles used by the wasps is different between treatments. However, our results are similar to what has previously been found in cultivated *B. oleracea* using the same aphid, lepidopteran and parasitoid species, also looking at the systemic effects of aphid induction (Soler *et al.*, 2012b). Though of a longer induction period than in this study, volatile profiles of single and dually infested plants could not be separated in the multivariate space, and likewise wasps did not differentiate between the two treatments, and this also at two different aphid densities.

When a plant is challenged by two attackers, multiple spatial and temporal effects may affect the level of impact that these antagonists may have on plant-mediated interactions with natural enemies (Dicke *et al.*, 2009). The effect of multiple attack may be dependent on such factors as order of arrival, density or spatial distribution on the plant. The spatial distribution of the attackers on the plant may be a crucial factor in determining the presence or absence of interference in indirect defenses. While our study examined the systemic effects of the initial inducer by placing caterpillars on a leaf above the initial inducer, research tends to point to stronger effects at a local level, where signaling pathway crosstalk becomes apparent. Application of *P. brassicae* egg extract on Arabidopsis leaves has been shown to lead to a local accumulation of SA, which then led to the suppression of caterpillar-induced JA pathway-dependent genes, yet this effect was not found in the distal plant tissues (Bruessow *et al.*, 2010). Likewise, aphids have stronger effects locally than systemically, as in Arabidopsis, SA-pathway associated genes were shown to be primarily expressed in the infested leaves (Moran & Thompson, 2001; De Vos *et al.*, 2005). Such a localized response to SA-inducing organisms may be a more general mechanism, as has also been demonstrated with pathogen infection, where defense trade-offs only occur when the biotrophic and necrotrophic pathogens infect the same leaf (Spoel *et al.*, 2007).

Nevertheless, the somewhat limited number of available studies on volatile emissions and natural enemy attraction in the context of dual attack show that it is quite common for the third trophic level to not be negatively affected by the presence of a non-host. De Rijk, Dicke & Poelman (2013) found that, out of 20 studies investigating carnivore preference when offered host-infested plants against plants infested with hosts and a non-host, only in 6 cases did the simultaneous presence of a non-host have a negative effect (for instance Rasmann & Turlings, 2007; Zhang *et al.*, 2009; Zhang *et al.*, 2013). In the majority of cases, when such dually challenged plants are offered against a plant with hosts only, enemies are frequently unaffected by, or even prefer dually infested plants both when the non-host is another herbivore (Moayeri *et al.*, 2007; De Boer *et al.*, 2008; Erb *et al.*, 2010; Bukovinszky *et al.*, 2012) or a plant pathogen (Cardoza *et al.*, 2003b; Rostás *et al.*, 2006). This, in combination with our results, suggests that overall the tritrophic cue appears quite robust, despite the potential for interference of the second attacker in the volatile composition.

The ecology of *C. glomerata* may also provide some further explanation. In the Netherlands the main lepidopteran hosts of this wasp, *P. brassicae* and *P. rapae* feed on a wide range of brassicaceous plants, both wild and cultivated (Fei *et al.*, 2014). Having several generations per year, they will be present on different host plants as the growth season progresses. It is, therefore, crucial for the parasitoids to be able to perceive host-induced plant volatiles from a wide range of plants (Gols *et al.*, 2012), and so they are likely responding to more general volatile cues and may not be so sensitive to small changes in the volatile blend during multiple attack, as observed in our study. Previous work with *C. glomerata* shows that they may not differentiate when having to choose between volatiles of plants with only hosts and plants also infested with a non-host caterpillars (Shiojiri *et al.*, 2000; Vos *et al.*, 2001) or even root herbivores (Soler *et al.*, 2007). However, *C. glomerata* has also been shown to discriminate between subtle differences in volatiles blends, for example between host-infested plants originating from different populations (Gols *et al.*, 2009).

In summary, we have shown that *C. glomerata* wasps are attracted to those plants that harbor their caterpillar host, regardless of the presence, or identity, of a non-host attacker infesting the plant prior to the arrival of caterpillars. Furthermore, plant volatile data support these results as the profiles of all treatments infested with caterpillars separate from those without, also irrespectively of initial attacker identity during dual attack. Together, our data strongly indicate that the host-induced volatile signal in *B. nigra* that attracts *C. glomerata* is robust and resists interference by non-host attackers, despite the induction of opposing defense signaling pathways by two of the studied

additional attackers. However, volatiles emitted by plants induced by non-hosts are also attractive to *C. glomerata*. As natural selection is predicted to act strongly on host-finding efficiency in parasitoids, the results of this study and others suggest that volatile-mediated foraging should be labile when the volatile signal is not a reliable indicator of the attacking species or when the wasp is not able to distinguish subtle differences in volatile blends. Alternatively, wasps do not only rely on these plant volatiles, but also use other cues, such as host-related products and visual cues during foraging, which in combination guide the wasps to their hosts. This gives valuable insight in how a plant species reacts to different naturally encountered aggressors, and points to a lack of specificity in the defense response.

Acknowledgements

We thank Léon Westerd, Frans van Aggelen, André Gidding and Joop Woelke for culturing the insects, Corné Pieterse and Hans van Pelt for providing the pathogen and advising on the inoculation procedure, Dani Lucas-Barbosa for her valuable assistance and discussions about the volatile compounds, and Wageningen University & Research Center's experimental farm (Unifarm) for rearing the plants. This work, as part of the European Science Foundation EUROCORES Programme EuroVOL, was supported from funds by the Earth and Life Sciences Council of the Netherlands Organisation for Scientific Research.

Supporting information

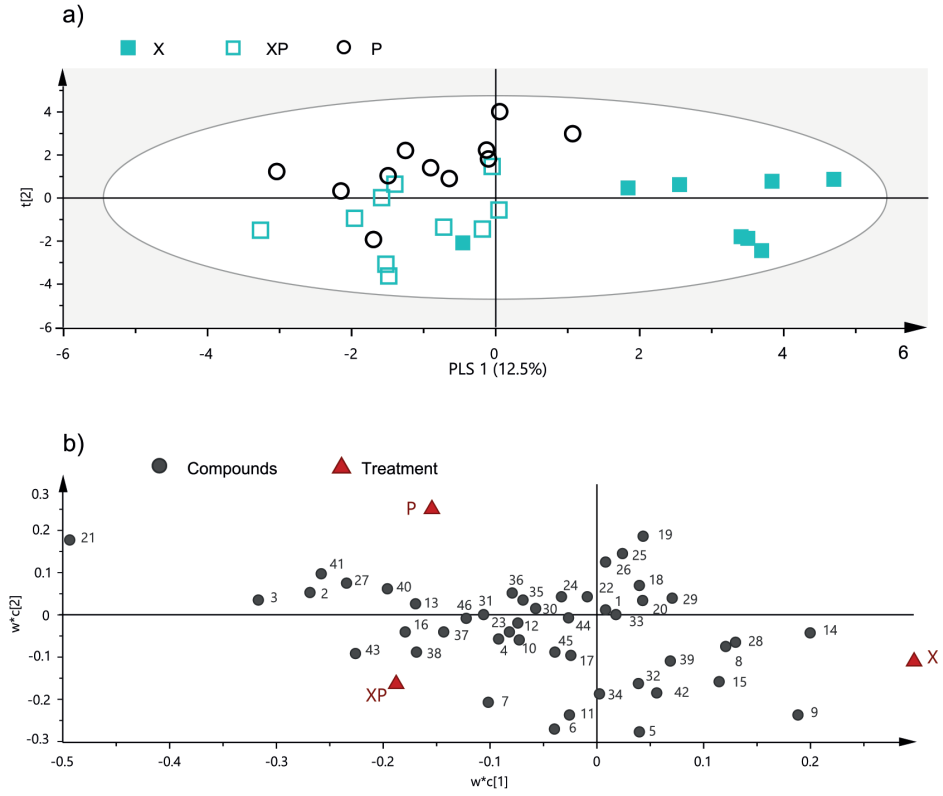


Figure S1. PLS-DA comparison of the volatile compounds emitted by individual *B. nigra* plants sampled after 72 h. (a) Score plot of the samples, with the percentage of explained variation in parentheses. Plants were infested with *P. brassicae* caterpillars (P), infected with *Xanthomonas* pathogen (X) or pathogen and caterpillars (XP). The PLS-DA resulted in a model with one significant component: $R^2X=0.125$ $R^2Y=0.329$ $Q^2=0.145$ (the second axis is shown for representational purposes). The ellipse defines the Hotelling's T^2 confidence region (95%). (b) Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 2.

Chapter 4

Volatile-mediated foraging behavior of three parasitoid species under conditions of dual insect herbivore attack

Camille Ponzio [1], Pasquale Cascone [2], Antonino Cusumano [3]*, Berhane T. Weldegergis [1], Nina E. Fatouros [1, 4] +, Emilio Guerrieri [2], Marcel Dicke [1] & Rieta Gols [1]

[1] Laboratory of Entomology, Wageningen University, P.O. Box 16, 6700 AA Wageningen, The Netherlands

[2] Institute for Sustainable Plant Protection, National Research Council of Italy, Portici, Italy

[3] Department of Agricultural and Forest Sciences, University of Palermo, Palermo, Italy

[4] Institute of Biology, Dahlem Centre of Plant Sciences, Freie Universität Berlin, Berlin, Germany

* Current address: Laboratory of Entomology, Wageningen University, P.O.Box 16, 6700 AA Wageningen, The Netherlands

+ Current address: Biosystematics Group, Wageningen University, P.O. Box 16 6700 AA Wageningen, The Netherlands

Published in a modified form in Animal Behaviour, 111, 197–206 (**2016**)

ABSTRACT

Infochemicals play an important role in structuring intra- and interspecific interactions. Many parasitoid wasp species rely on herbivory or oviposition-induced plant volatiles (HIPVs/OIPVs) to locate their herbivorous hosts, and must cope with variation in the volatile blends due to factors such as plant/host species, herbivore density or attack by several herbivores. However, little is known about how dual herbivory or changes in herbivore density affect multiple parasitoid species, each attacking a different herbivore, in the same system. In a natural system, we investigated the effect of dual attack on the ability of three parasitoid species to differentiate between volatiles induced by hosts and those induced by a combination of hosts and non-hosts. Black mustard, *Brassica nigra*, plants were infested with eggs or caterpillars of *Pieris brassicae*, alone or in combination with different densities of *Brevicoryne brassicae* aphids. We determined the ability of three different parasitoid species that parasitize either *P. brassicae* eggs (*Trichogramma brassicae*), caterpillars (*Cotesia glomerata*) or *B. brassicae* aphids (*Diaeretiella rapae*) to discriminate between the induced volatiles, and analyzed the plant volatile blends. Dual infestation did not affect the parasitoid species equally and aphid infestation altered, in a density-dependent manner, the volatile-mediated foraging of all three parasitoid species. Chemical analyses of the volatile blends revealed nonlinear emission patterns in relation to aphid density in both plants attacked by aphids alone and in plants attacked by a combination of aphids and caterpillars. Simple correlations between behavior and volatile emissions in pairwise comparisons suggest the importance of certain volatiles explaining attraction, whereas dose-response type analyses reveal that these simple correlation analyses provide an incomplete picture.

Keywords: herbivore-induced plant volatiles, indirect defense, multiple attack, multitrophic interactions, oviposition-induced plant volatiles.

Introduction

Chemical cues are an important source of information for individuals and mediate ecological interactions, whether within or between species, across the taxonomic spectrum, covering organisms such as microorganisms, plants, insects and mammals. (Hildebrand, 1995; Dicke & Grostal, 2001; Cardé & Millar, 2004) Within a species, these chemical cues can indicate kinship, location, health status, fertility (via sex pheromones), territory, or inform others of danger (alarm pheromones). However, these chemical cues can be exploited by members of a different species, for instance to locate prey (Wyatt, 2003). This makes chemical information an important element in structuring individual behavior and the interactions between community members (Vet, 1996; Stam *et al.*, 2014). Volatile chemicals emitted by plants have a wide range of functions, such as plant-to-plant signals, defense chemicals or pollinator attractants (Holopainen, 2004; Peñuelas & Llusià, 2004; Heil & Karban, 2010), and so a volatile blend can have different meanings for different organisms.

For insects, these volatiles may indicate a suitable food source, an oviposition site, the presence of herbivorous prey or hosts, or they may be used by pollinators to locate flowering plants (Vet & Dicke, 1992; Pichersky & Gershenzon, 2002; Bruce *et al.*, 2005; Lucas-Barbosa *et al.*, 2011). Parasitoid wasp species that attack insect herbivores rely on herbivore or oviposition-induced plant volatiles (HIPVs/OIPVs) to locate host-infested plants (Mumm & Dicke, 2010; Hilker & Fatouros, 2015), and even identify the most suitable host developmental stages (Takabayashi *et al.*, 1995; Pashalidou *et al.*, 2014). However, in order to successfully find these plants they must deal with a wide range of variation in the volatile blend emitted by the plants (Vet & Dicke, 1992; Clavijo McCormick *et al.*, 2012), which may not always be reliable indicators of herbivore identity and host suitability (Vos *et al.*, 2001; De Rijk *et al.*, 2013 and references within). The herbivore-specific variation in HIPV blends has been attributed to the different elicitors contained in the herbivore oral secretions that differentially affect phytohormonally regulated plant responses and eventually volatile emission.

Moreover, in nature plants are commonly challenged by more than one attacker, either simultaneously or sequentially, potentially compromising volatile-mediated foraging of natural enemies of the herbivores (Dicke *et al.*, 2009). Multiple attack adds a further layer of complexity to the interactions within a tritrophic system, as non-host attackers can have a significant impact on the foraging behavior of parasitoid wasps (Rodriguez-Saona *et al.*, 2005; De Rijk *et al.*, 2013; Ponzio *et al.*, 2013). Non-host identity can have an effect via crosstalk between different defense signaling pathways or through attacker-specific

interactions with the plant (De Vos *et al.*, 2005; Stam *et al.*, 2014). A myriad of additional factors can come into play, such as the location of the different attackers on the plant, the timing of the attacks or herbivore density (Dicke *et al.*, 2009; Zhang *et al.*, 2009; Kroes *et al.*, 2015).

Many of the studies examining the effects of dual attack on parasitoid foraging look at how different non-host herbivores affect one focal member of the third trophic level, but less is known about how one given dual attack scenario may affect multiple species at the third trophic level. When two tritrophic systems are combined on the same plant, dual attack can differentially affect the respective parasitoid species of the attackers that may not interpret the volatile cues in the same manner (Shiojiri *et al.*, 2000), and this may be especially true depending on whether the attacking herbivores induce similar or opposing defense signaling pathways. In this context, and in a natural biological system, we examined the effect of dual infestation with aphids and lepidopteran eggs or caterpillars on the attraction of parasitoid species respectively attacking the aphids, eggs or caterpillars (Fig. 1). In addition, we determined the effect of aphid density on volatile-mediated foraging behavior. Black mustard, *Brassica nigra* (Brassicaceae) plants were infested with eggs or early first-instar caterpillars of the large cabbage white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), alone or in combination with *Brevicoryne brassicae* (Hemiptera: Aphididae) aphids. We predicted that the volatile blend that is produced in response to dual infestation would be less attractive to the respective parasitoids of the two inducing herbivore species than the blend that is emitted by plants infested by hosts alone. Moreover, behavioral interference was predicted to be stronger for the parasitoid of the eggs and the caterpillars with increasing aphid density, but to be attenuated for the aphid parasitoid. Using wind tunnel and olfactometer bioassays, we compared the effects of dual attack on the host-finding behavior of three different parasitoid species that parasitize either the eggs (*Trichogramma brassicae*), caterpillars (*Cotesia glomerata*) or aphids (*Diaeretiella rapae*). The composition of the induced plant volatile blends was also analyzed, to assess whether changes in the blend characteristics could explain the observed wasp behavior. The herbivores were chosen on the basis of the defense signaling pathways they induce: aphids and eggs primarily induce salicylic acid-mediated defenses, while defenses against caterpillars are largely induced via the jasmonic acid signaling pathway (Bruessow *et al.*, 2010; Kroes *et al.*, 2015).

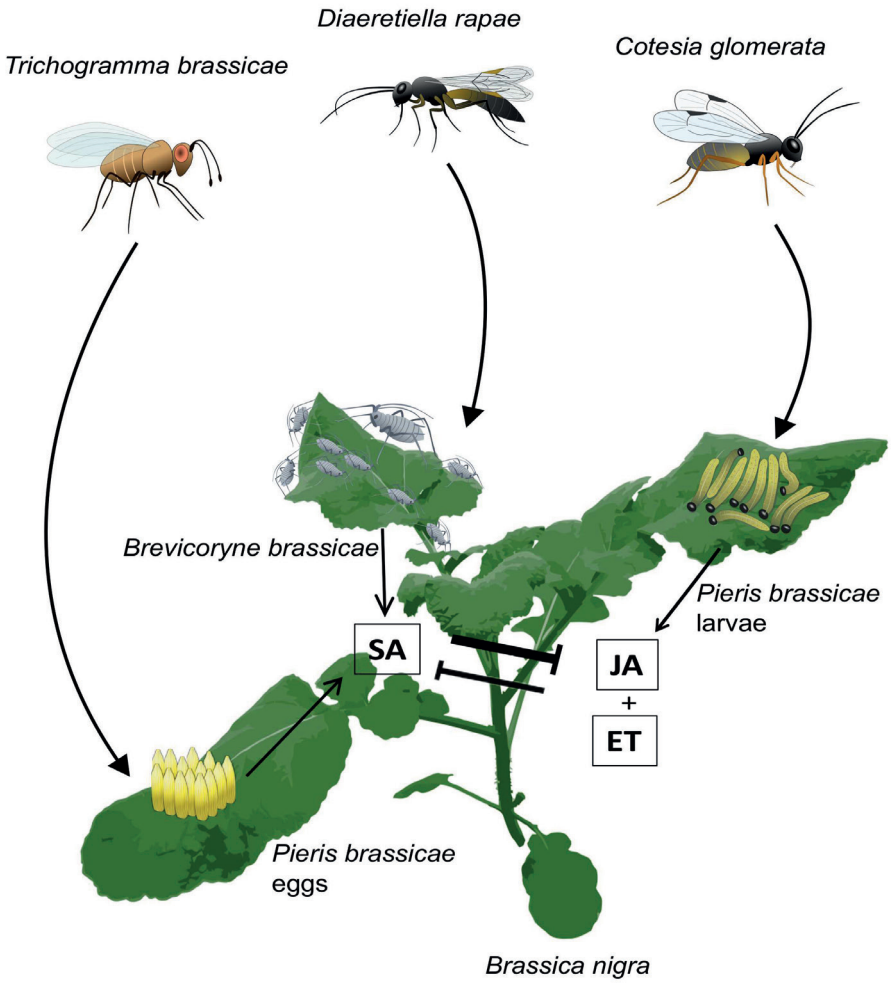


Figure 1. Illustration of the three insect or egg attackers and their respective parasitoid species used in this study. The herbivore attackers primarily induce either the salicylic acid defense pathway (SA) or the jasmonic acid (JA) and ethylene (ET) pathways, which frequently have inhibitory effects on each other (symbolized by lines capped with bars).

Methods

Plant and insect material

Brassica nigra plants, originating from seeds collected from a wild population growing in Wageningen (The Netherlands), were cultivated under greenhouse conditions at 22 ± 2 °C, with a relative humidity (RH) of 60-70% and a light:dark (LD) regime of 16:8 h. Three

naturally occurring insect attackers were used, with their associated natural enemy (Fig. 1). *Pieris brassicae* larvae and adults and *B. brassicae* aphids were reared on Brussels sprouts plants, *Brassica oleracea* var. *gemmifera* cv. Cyrus, in a climate-controlled room (21 ± 1 °C, 50-70% RH, 16:8 h LD). The specialist larval parasitoid, *C. glomerata*, was reared from *P. brassicae* under similar conditions. Cocoons were kept in a (30 x 30 x 30 cm) mesh cage (Bugdorm, Taiwan) and adults were supplied with a 6% sucrose solution and honey. Three-to-seven-day-old, mated female wasps were used in the bioassays. The generalist egg parasitoid *Trichogramma brassicae* was reared from eggs of *Ephestia kuehniella* moths (Koppert Biological Systems, The Netherlands) in a climate chamber at 25 ±1 °C, 50-70% RH and 16:8 h LD. Two-to-five-day-old, mated females were used in all experiments. The generalist aphid parasitoid, *D. rapae*, was reared in a climate cabinet (20 ± 1 °C, 50-70% RH, 16:8 h LD) from *Myzus persicae* aphids feeding on radish, *Raphanus sativus* cv. Gaudry, plants. Adults were supplied with water and honey. Mummies were collected and placed in a separate cage prior to emergence and kept under similar conditions. Two-to-four-day-old, mated female wasps were used in the bioassays. All wasps of the three species used in the experiments were naïve, i.e. they had no previous contact with plant material, their hosts or host products during their adult life.

Plant treatments

All experimental plants were kept in 35 x 35 cm and 60 cm high mesh cages (Vermandel, The Netherlands), with each treatment in a separate cage. For treatments with a single herbivore, plants were infested with either 25, 50 or 100 early instar *B. brassicae* nymphs, 20 first-instar *P. brassicae* caterpillars or one clutch (on average about 30 eggs) of *P. brassicae* eggs. Twenty caterpillars were used in order to match the density used in previous experiments (Ponzio *et al.*, 2014). All feeding insects, or eggs, were placed on the youngest fully expanded leaf. Eggs were obtained by placing the *B. nigra* plants in a cage containing >100 *P. brassicae* butterflies. The plant was covered with a mesh bag with the desired leaf protruding, and butterflies were allowed to oviposit until the desired number of eggs was obtained, which takes 10-20 min. For treatments with dual stress (eggs and aphids, or aphids and caterpillars), plants with aphids or eggs were prepared as described above. In the case of dual infestation with aphids and caterpillars, 48 h after infestation with the aphids 20 first-instar *P. brassicae* caterpillars were added on the same leaf, and allowed to feed for a further 24 h. For plants dually infested with eggs and aphids, the aphids were placed on the plant immediately after oviposition by *P. brassicae* butterflies. All plants were infested for a total duration of 72 h (or 24 h for plants infested with caterpillars only) before being tested in the wind tunnel or Y-tube olfactometer. *Pieris*

brassicae eggs hatch only after 96 h at 21 °C. Healthy, uninfested plants were kept under the same conditions as the treated plants.

Wind tunnel bioassays

Plant preference of the parasitoid *C. glomerata* was tested in a wind tunnel setting as previously described by Geervliet et al. (1994), at 25 ± 1 °C, 60 ± 10% RH and with a wind speed of 10 cm/s. Wasp landing preference was recorded for seven different treatment combinations: (1) and (2): healthy plant versus plant infested with either 50 or 100 aphids; (3): healthy plant versus plant infested with 20 caterpillars; (4) and (5): healthy plant versus plant infested with 50 or 100 aphids, respectively, and 20 caterpillars; (6) and (7): plant infested with 20 caterpillars versus one of the dually infested treatments at the two different aphid densities. Plants were placed in the wind tunnel 30 min before the start of the bioassays to allow for the dissipation of volatiles resulting from handling. Wasp manipulation and release were carried out as described by Ponzio *et al.* (2014). Females were released in two to three groups of five individuals. The pooled response of these wasps served as one data point and this was repeated nine to 11 times with new wasps and newly prepared plants for each tested plant combination. Wasps that did not respond within the observation period set at 15 min were scored as nonresponding and excluded from the statistical analysis. To exclude any potential bias, the positions of the plants were exchanged after each group of released females.

Y-tube olfactometer bioassays

Bioassays with both *T. brassicae* and *D. rapae* wasps were conducted in a dynamic airflow Y-tube olfactometer described in detail by Fatouros *et al.* (2012). Pressurized air was filtered through activated charcoal and humidified by passing through a jar with water. Incoming airflow was regulated by a flowmeter at 200 ml/min. The airflow was then divided into two sub flows that each fed into a glass jar containing an odor source. Air from each jar then flowed into one of the arms of a glass Y-tube olfactometer, with an airflow of 100 ml/min.

Ten female wasps were released into the system simultaneously, with two groups released per tested plant pair. The positions of the plants were swapped between releases of the two groups. It was previously established that group release in a Y-tube olfactometer did not influence the behavior of wasps (Fatouros *et al.*, 2012). For the statistical analysis, the groups were then pooled for each tested plant pair. After 15 min (for *D. rapae*) or 30 min (for *T. brassicae*) their preferred odor source was recorded, with a choice considered

to be made when wasps were found inside the trapping bulbs, located near the ends of the Y-tube arms. Wasps that did not respond within the observation period were scored as nonresponding and were excluded from the statistical analysis. The same treatment combinations as for the bioassays with *C. glomerata* were tested, with some differences: for the bioassays with *T. brassicae*, plants were infested with eggs instead of caterpillars, and for *D. rapae* an additional three test combinations, with 25 aphids (control versus 25 aphids, control versus caterpillars plus 25 aphids, and 25 aphids versus caterpillars plus 25 aphids), were included to further study the effect of aphid density on wasp odor preference. Eight replicates were tested per plant treatment pair for both wasp species.

Headspace collection and analysis

Volatiles were collected from plants of all the different treatments used in the behavior assays. The plants were prepared as described in the plant treatment section, with nine replicates per treatment. Trapping of the volatiles and their subsequent analysis via GC-MS were carried out as described in detail by Ponzio *et al.* (2014), with some minor modifications. Volatile collection was done with synthetic air (Air Synthetic 4.0 Monitoring from Linde Gas, Schiedam, The Netherlands) as the use of pure air with no impurities is better suited for volatile trapping, and is commonly used for gas chromatography. Additionally, a ZB-5MSi analytical column (30 m x 0.25 mm internal diameter x 0.25 μm film thickness with 5 m built-in guard column, Phenomenex, Torrance, CA, U.S.A.) was used, and the gas chromatograph oven program was as follows: the temperature was initially held at 40 °C for 2 min, raised at 4 °C/min to 220 °C and then was immediately raised at 10 °C/min to a final temperature of 280 °C, where it was kept for 7 min under a helium flow of 1 ml/min in a constant flow mode.

Statistical analysis

To investigate whether parasitoid preferences differed when various combinations of plant treatments were offered, the data were analyzed using logistic regression in SAS version 9.2 (SAS Institute Inc. Cary, NC, U.S.A.) with plant treatment as a fixed factor. For cases of overdispersion, we corrected for this by allowing the variance functions of the binomial distribution to have a multiplicative overdispersion factor. In the comparison with control plants, the number of wasps choosing the attacker-infested plants out of the total number of responding wasps was entered as the response variable. In the analysis of dual versus single attack, the number of wasps choosing the dually infested plant out of the total number of responding wasps was entered as the response variable. Each bioassay

with one pair of plants served as a replicate. To determine within each comparison whether there was a significant preference for one of the offered plant treatments, we tested H_0: logit=0, which equals testing equal preference for P=0.5.

The volatile emission patterns, measured as peak areas divided by the fresh mass of the plant, were analyzed through multivariate data analysis using PSL-DA (projection to latent structures discriminant analysis; (Eriksson *et al.*, 2006). The analysis additionally shows the variable importance in the projection (VIP) of each variable (in this case, the different compounds), with variables having VIP values greater than 1 being most influential in the model (Eriksson *et al.*, 2006). To directly link behavior and chemistry, PLS-DA models were run including the two test treatment groups. Here, only those compounds having a VIP value greater than 1 were included, to explicitly focus on the most important compounds. In all cases, data were log-transformed, mean-centered and scaled to unit variance before they were subjected to the analysis.

Results

Attraction of parasitoids to induced plant volatiles

Cotesia glomerata: parasitoid of P. brassicae caterpillars

In the wind tunnel experiments using *C. glomerata*, significant differences were observed between the different treatment pairs, in tests of both healthy versus infested plants (generalized linear model, GLM: χ^2_4= 175, P <0.001) and host-infested versus dually infested plants (GLM: χ^2_1= 22, P <0.001; Fig. 2a). Wasps preferred volatiles from host-infested plants to volatiles from healthy plants, with neither aphid presence nor their density affecting wasp attraction to host-infested plants (Fig. 2a). However, when plants infested with aphids alone were offered against healthy plants, density-dependent effects were present. Wasps equally landed on healthy plants and those infested with 50 aphids, while volatiles from plants with 100 aphids were significantly less preferred than healthy plants. When dually infested plants were offered against host-infested plants, clear density-dependent effects of the aphids on wasp foraging were recorded. Volatiles from plants dually challenged with 50 aphids and caterpillars were more attractive than volatiles from plants with caterpillars only, but when the density was increased to 100 aphids, there was a switch in preference, with volatiles of dually infested plants becoming significantly less attractive than those of plants with only caterpillars.

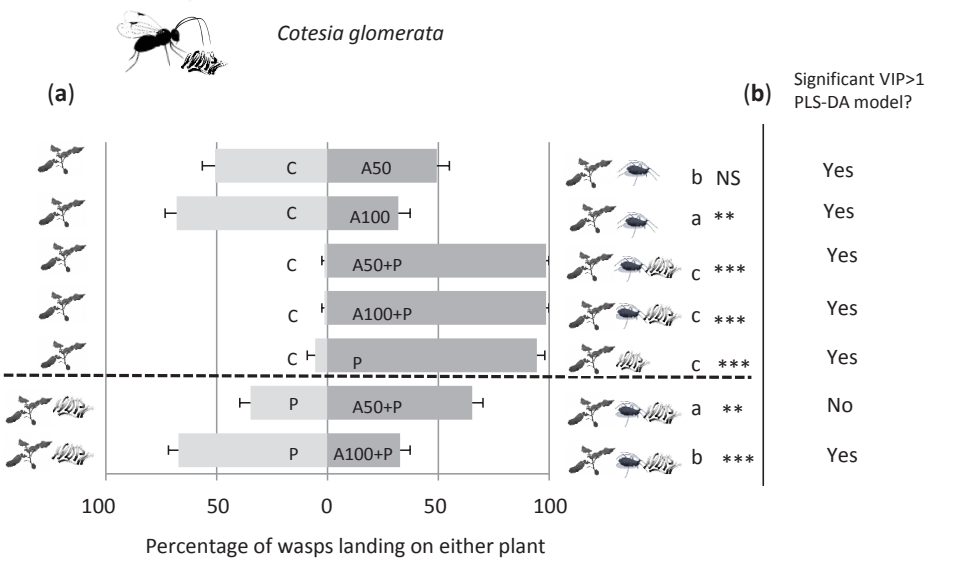


Figure 2. (a) Preference of *C. glomerata* wasps (caterpillar parasitoids) in a two-choice set-up in a wind tunnel (percentage ± SE, plant *N*=10) where healthy control plants (C) or plants infested with 20 *P. brassicae* caterpillars (P) were tested against a plant infested with one of the following: single infestation with 20 caterpillars (P), 50 (A50) or 100 (A100) *B. brassicae* aphids, or dual infestation of 20 caterpillars with 50 (A50+P) or 100 (A100+P) aphids. Significant differences between treatments ($P < 0.05$) are indicated with different letters, with the treatment pairs below the dashed line analyzed separately from those above it. Asterisks indicate a preference that is significantly different from a 50:50 distribution within a choice test: ** $P \leq 0.01$; *** $P \leq 0.001$. (b) Results of pairwise PLS-DA models, comparing the same treatment pair as for the behavioral study, which indicates whether the models result in at least one significant principal component (PC). Models included only those compounds that have a variable importance in projection (VIP) value greater than 1, i.e. compounds that contribute the most to the model.

Trichogramma brassicae: parasitoid of P. brassicae eggs

In Y-tube olfactometer experiments with *T. brassicae*, preference differed between the different tested plant combinations, but only for healthy versus infested plant combinations (healthy versus infested: GLM: $\chi^2_4 = 21$, $P < 0.001$; host-infested versus dually infested: GLM: $\chi^2_1 = 0.2$, $P = 0.66$; Fig. 3a). While volatiles induced by an infestation of only host eggs were preferred over those emitted by healthy plants (Fig 3a, comparison C versus E), the simultaneous presence of aphids and host eggs interfered with normal foraging behavior to a certain extent, as the wasps no longer distinguished volatiles from plants infested with eggs and aphids from volatiles emitted by healthy plants (Fig 3a, comparison C versus A50+E, C versus A100+E). This was apparent at both aphid densities; however, there was no additional effect of aphid density. The effect is relatively subtle; when behavioral responses of the wasps to the different treatment pairs were compared, the strength of preference for dually infested or egg-only infested plants was similar. In the

absence of eggs, in tests of aphid-infested plants against healthy plants there were no density-dependent effects, although a negative trend was visible. When the dually infested treatments were offered against plants with eggs only, at both densities the wasps displayed a preference for volatiles emitted by plants infested with only eggs.

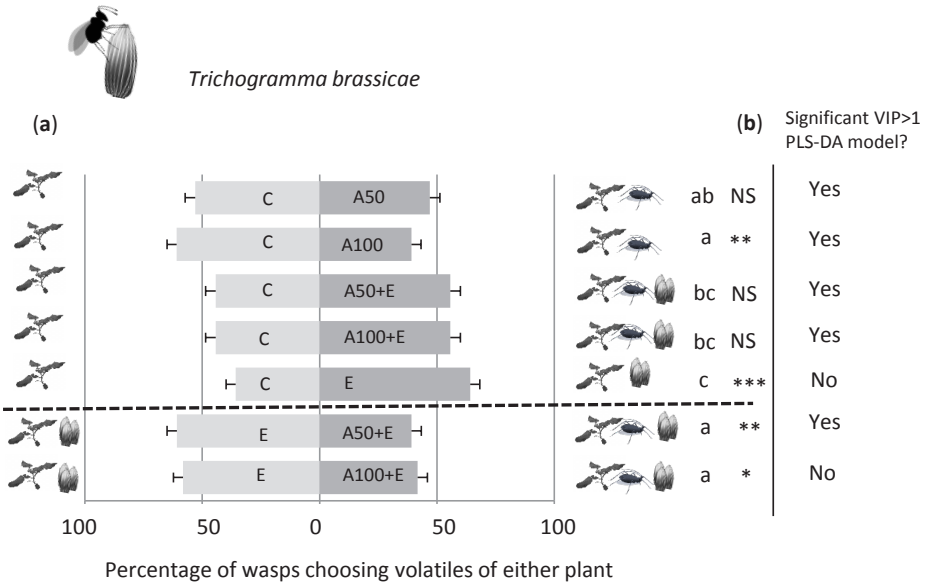


Figure 3. (a) Preference of *T. brassicae* wasps (egg parasitoids) in a two-choice set-up in a Y-tube olfactometer (percentage ± SE, plant $N = 8$) where healthy control plants (C) or plants infested with 30 *P. brassicae* eggs (E) were tested against a plant infested with one of the following: single infestation with 30 eggs (E), 50 (A50) or 100 (A100) *B. brassicae* aphids, or dual infestation of 30 eggs with 50 (A50+P) or 100 (A100+P) aphids. Significant differences between treatments ($P < 0.05$) are indicated with different letters, with the treatment pairs below the dashed line analyzed separately from those above it. Asterisks indicate a preference that is significantly different from a 50:50 distribution within a choice test: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. (b) Result of pairwise PLS-DA models, comparing the same treatment pair as for the behavioral study, which indicates whether the models result in at least one significant principal component (PC). Models included only those compounds that have a variable importance in projection (VIP) value greater than 1.

Diaeretiella rapae: parasitoid of B. brassicae aphids

For *D. rapae* there was also a significant difference in preference between the different test combinations, but only in the case of healthy versus infested plants (GLM: $\chi^2_6 = 37$, $P = 0.0001$) but not for host-infested versus dually infested plants (GLM: $\chi^2_2 = 0.6$, $P = 0.74$; Fig. 4a). Interestingly, a negative correlation was found between parasitoid attraction and aphid density. This was the case when healthy plants were tested against aphid-infested plants, and also against plants infested with both aphids and caterpillars. When testing each aphid density against the associated dual infestation, the presence of caterpillars did

not influence plant preference at any of the three aphid densities, as the wasps did not significantly prefer one treatment over the other.

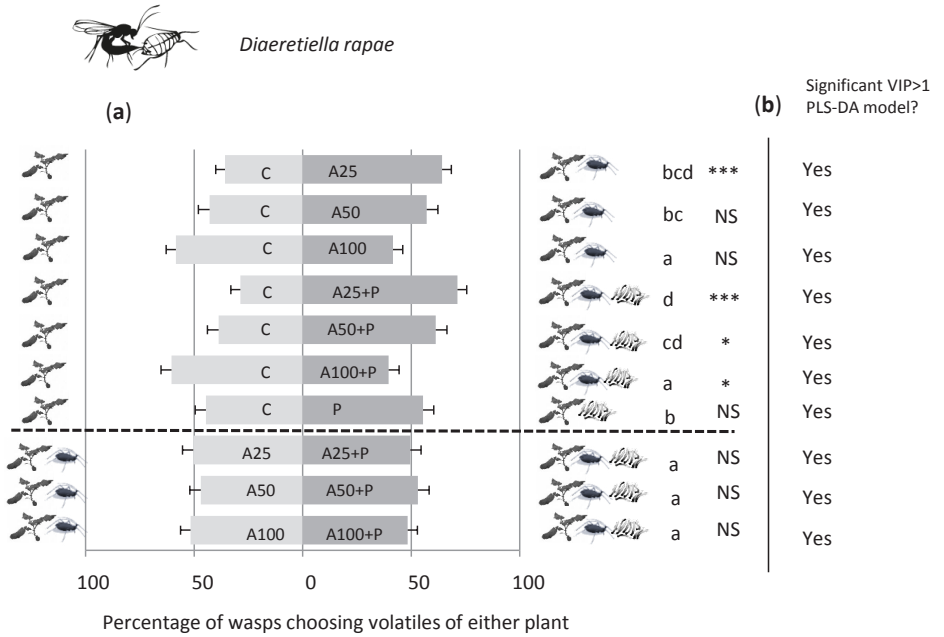


Figure 4. (a) Preference of *D. rapae* wasps (aphid parasitoids) in a two-choice set-up in a Y-tube olfactometer (percentage ±SE, plant *N*=8) where healthy control plants (C) or plants infested with 20 *P. brassicae* caterpillars (P) were tested against a plant infested with one of the following: single infestation with 20 caterpillars (P), 25 (A25), 50 (A50) or 100 (A100) *B. brassicae* aphids, or dual infestation of 20 caterpillars with 25 (A25+P), 50 (A50+P) or 100 (A100+P) aphids. Significant differences between treatments ($P < 0.05$) are indicated with different letters, with the treatment pairs below the dashed line analyzed separately from those above it. Asterisks indicate a preference that is significantly different from a 50:50 distribution within a choice test: * $P \leq 0.05$; *** $P \leq 0.001$. (b) Result of pairwise PLS-DA models, comparing the same treatment pair as for the behavioral study, which indicates whether the models result in at least one significant principal component (PC). Models included only those compounds that have a variable importance in projection (VIP) value greater than 1.

Induced plant volatiles

A total of 37 different volatile compounds were detected in the headspace of the plants. Plants of the 11 different treatments all emitted the same compounds, but in different proportions (Supporting Information Table S1). A PLS-DA of the samples from all treatments with either aphids, caterpillars or a combination of both resulted in a model with one significant principal component (PC; Fig. 5). This PC separated the treatments based on the presence or absence of caterpillars. Further models were made to see whether any further separation could be found in these two groups. When comparing the treatments containing only aphids, a significant PC was found which distinguished plants infested

by 50 aphids from the plants that were infested with either 25 or 100 aphids (Fig. 6). No other significant PCs could be found when comparing samples with 25 or 100 aphids, nor was there any separation between the treatments that included caterpillars. Analysis of the samples from aphid-infested, egg-infested and the matching dually challenged plants did not result in a significant model (model not shown), indicating a strong similarity in the volatile blends induced by eggs or by aphids.

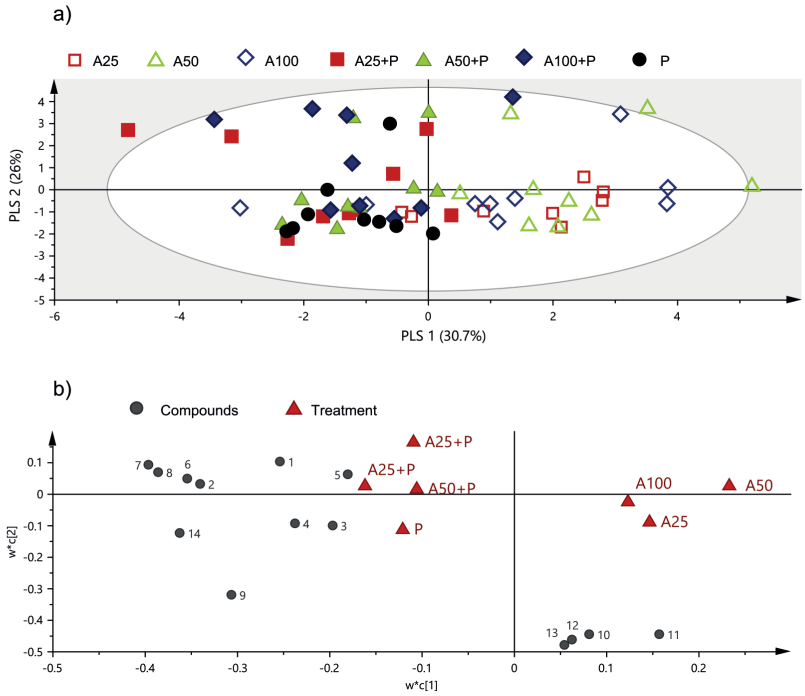


Figure 5. PLS-DA comparison of the volatile compounds emitted by single or dual infested individual *B. nigra* plants sampled after 72 h. (a) Score plot of the samples, with the percentage of explained variation in parentheses. Plants were infested with 25, 50 or 100 *B. brassicae* aphids (A25, A50, A100), 20 *P. brassicae* caterpillars (P) or a combination of each aphid density and caterpillars (A25+P, A50+P, A100+P). The PLS-DA resulted in a model with one significant component: $R^2X = 0.308$, $R^2Y = 0.0914$, $Q^2 = 0.069$ (the second axis is shown for representational purposes). The ellipse defines the Hotelling's T2 confidence region, which is a multivariate generalization of the Student's *t* test and gives a 95% confidence interval for the observations. (b) Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds towards the model. Only compounds having a variable importance in projection value greater than 1 were included. Compound identity is as follows: (1) 2-butenenitrile, (2) 3-butenenitrile, (3) 1-penten-3-ol, (4) 3-pentanone, (5) 2-methylbutanenitrile, (6) (Z)-3-hexen-1-ol, (7) allyl isothiocyanate, (8) (Z)-3-hexen-1-ol acetate, (9) (E)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT), (10) 7-β-H-silphiperfol-5-ene, (11) unknown sesquiterpene, (12) silphiperfol-6-ene, (13) tricyclo[6.3.0.0(1,5)]undec-2-en-4-one, 2,3,5,9-tetramethyl (TUT), (14) total volatiles.

In addition, pairwise PLS-DA comparisons were done for all the treatment combinations tested in the behavioral assays, and for these the models included only compounds having

a VIP value greater than 1, i.e. those compounds that contribute most strongly to the model. When comparing healthy plants against one of the single or dual infestation treatments, the pairwise comparisons nearly all resulted in a significant model (Figs 2b, 3b and 4b), with the exception of the treatment pair healthy plants versus egg-infested plants in the bioassays with *T. brassicae* (Fig. 3b). When comparing host-infested and dually infested treatments, significant models were found for five of the seven pairwise comparisons, also in instances where there was no behavioral discrimination (e.g. Fig. 4). The volatile blends of caterpillar-infested plants and plants dually infested with 50 aphids were not significantly different from each other (Fig. 2b), and this was also the case for the comparison between egg-infested plants and plants with eggs plus 100 aphids (Fig. 3b).

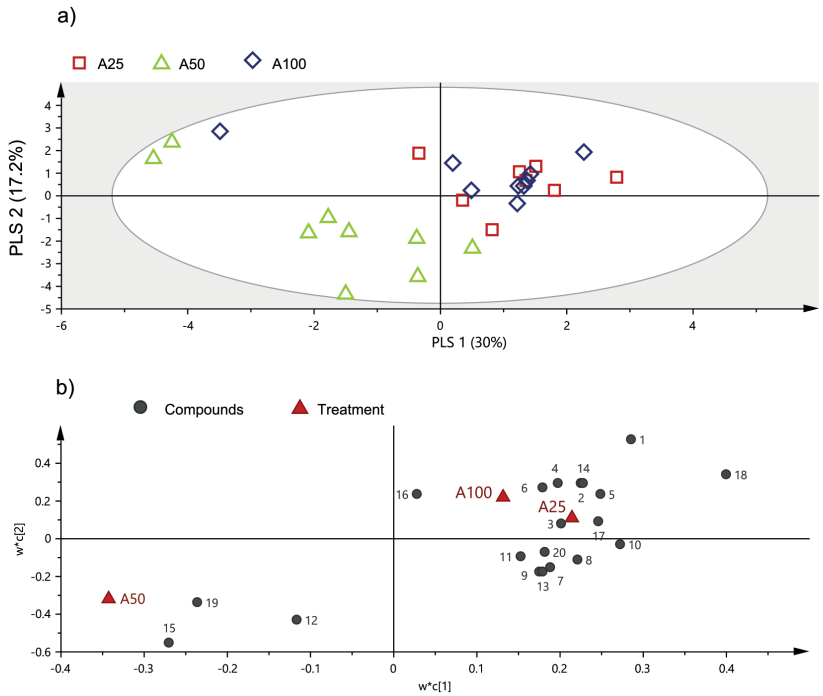


Figure 6. PLS-DA comparison of the volatile compounds emitted by individual aphid-infested *B. nigra* plants sampled after 72 h. (a) Score plot of the samples, with the percentage of explained variation in parentheses. Plants were infested with 25 (A25), 50 (A50) or 100 (A100) *B. brassicae* aphids. The PLS-DA resulted in a model with two significant components: $R^2X = 0.3$, $R^2Y = 0.229$, $Q^2 = 0.062$ and $R^2X = 0.172$, $R^2Y = 0.172$, $Q^2 = 0.107$. The ellipse defines the Hotelling's T2 confidence region. (b) Loading plot of the first two components of the PLS-DA, showing the contribution of each of the compounds towards the model. Only compounds having a variable importance in projection value greater than 1 were included. Compound identity is as follows: (1) 2-butenenitrile, (2) dimethyl disulphide, (3) (Z)-3-hexen-1-ol, (4) myrcene, (5) (Z)-3-hexen-1-ol acetate, (6) isomenthone, (7) 7-α-H-silphiperfol-5-ene, (8) presilphiperfol-7-ene, (9) 7-β-H-silphiperfol-5-ene ,(10) unknown sesquiterpene, (11) silphiperfola-5,7(14)-diene, (12) unknown sesquiterpene, (13) silphiperfol-6-ene, (14) α-funebrene, (15) longifolene, (16) unknown compound, (17) β-caryophyllene, (18) α-caryophyllene, (19) (*E,E*)-α-farnesene, (20) tricyclo[6.3.0.0(1,5)] undec-2-en-4-one, 2,3,5,9-tetramethyl (TUT).

Discussion

In this study we compared the effects of dual infestation with *B. brassicae* aphids in combination with *P. brassicae* eggs or caterpillars on the foraging behavior of their respective parasitoids. Our results show that dual infestation can affect volatile-mediated foraging; however, the effect of interference by the non-host attacker was dependent on the parasitoid species. For the aphid parasitoid *D. rapae*, there was no effect of dual attack by non-host caterpillars. For the egg parasitoid *T. brassicae*, the non-host aphids did interfere with foraging. Finally, for the larval parasitoid *C. glomerata*, dual infestation by non-host aphids also interfered with foraging, in a density-dependent manner. Furthermore, our results show a general negative, density-dependent effect of aphid infestation on volatile-mediated behavioral responses of natural enemy species, both when the aphids are the host and when they are the non-host herbivore of the parasitoid species in question. However, the behavior of the different wasp species was not affected in the same way by aphid infestation. When the alternative volatile source was a clean plant, the behavioral response was negatively correlated with aphid density for all three wasp species, including the aphid parasitoid *D. rapae*. In the presence of the non-host *P. brassicae* caterpillars, *D. rapae* responded similarly to aphid density whereas the other two parasitoid species, *C. glomerata* and *T. brassicae,* clearly preferred the plants with their host irrespective of aphid presence or aphid density. However, when the difference between the two volatile sources was more subtle, i.e. when the alternative volatile source was a host-infested plant, the latter two species responded differently. Although *T. brassicae* clearly preferred host (egg)-infested plants over dually infested plants, regardless of aphid density, *C. glomerata* preferred host (caterpillar)-infested plants over dually infested plants at a high aphid density, whereas the reverse result was found at a lower aphid density.

It is important for parasitoid wasps to be able to distinguish between plants infested with host and non-hosts, and to have limited interference from non-host presence. If a wasp cannot make the distinction, more time will be spent searching for host-infested plants, which may lead to a decrease in host finding efficiency, and so a lower number of offspring. Here, both the egg parasitoid *T. brassicae* and the larval parasitoid *C. glomerata* either preferred clean plants over aphid-infested plants or did not discriminate between these two plant types depending on aphid density. Furthermore, plants infested with hosts and aphids were clearly preferred over clean plants. These results suggest that there is no interference of non-host aphids with volatile-mediated foraging of these two parasitoid species. Previous studies have shown that naïve *C. glomerata* females cannot discriminate between volatile blends emitted by host and non-host caterpillar-infested

plants (Geervliet *et al.*, 1996; Shiojiri *et al.*, 2000; Bukovinszky *et al.*, 2012). Members of different insect feeding guilds, such as phloem-feeding aphids and tissue-chewing caterpillars, are known to activate different defense signaling pathways (De Vos *et al.*, 2005; Thaler *et al.*, 2012). This may explain why naïve *C. glomerata* distinguished between aphid- and caterpillar-induced volatile blends but not between those induced by different host and non-host caterpillar species. The foraging by egg parasitoids on plants infested by eggs plus non-host attackers is largely unexplored, and recent work addressing the question has also found that the presence of non-host herbivores can disrupt the attraction of an egg parasitoid to OIPVs (Moujahed *et al.*, 2014). It is noteworthy that the spatial distribution of the herbivores on the plant can also influence wasp foraging behavior. Aphid density-dependent effects are recorded when both herbivores infest the same leaf (this study) but not when, using the same plant-herbivore system, the herbivore species are feeding on separate leaves (Ponzio *et al.*, 2014). It is known from phytopathogen-based systems that defense trade-offs against two attackers are stronger locally than systemically (Spoel *et al.*, 2007), and such effects can probably be expected as well during insect attack (Dicke *et al.*, 1993; Mousavi *et al.*, 2013). Thus, the identity of the non-host, and the spatial distribution of host and non-host herbivores on the plant, can influence volatile-mediated foraging of a parasitoid (see also de Rijk et al. (2013) for further discussion). Rearing history may also be an additional factor to consider, as the different parasitoid hosts were not reared on the same plant species, and may have acquired different experiences during their larval or pupal stages. Given the variation in host specialization, it is likely that rearing history will influence the various parasitoids to different degrees. Moreover, in nature the parasitoids are also likely to develop from hosts feeding on different food plant species.

Foraging behavior of the aphid parasitoid *D. rapae* was not affected by the presence of caterpillar non-hosts in combination with host aphids, which confirms previous work on this parasitoid species in a different plant-non-host system (Agbogba & Powell, 2007). Most strikingly, however, and against prediction, the wasps were negatively affected by increasing aphid density despite the fact that *D. rapae* is an aphid parasitoid commonly found to parasitize *B. brassicae*. This parasitoid species attacks both generalist and specialist aphid species infesting brassicaceous plant species and is known to be attracted to isothiocyanates, whether of plant origin or applied as pure compounds onto plants (Titayavan & Altieri, 1990; Blande *et al.*, 2007; Pope *et al.*, 2008). These compounds are by-products resulting from the hydrolysis of glucosinolate plant secondary metabolites by myrosinase enzymes in the Brassicaceae (Fahey *et al.*, 2001), which are then mobilized in defense against attacking insect herbivores. Generalist species are usually more strongly

affected by these defense compounds than are specialists (Rask *et al.*, 2000). Differences in emission rates of this class of compounds could thus be expected to play a role in wasp foraging behavior. However, in our study, emission rates of allyl isothiocyanate, the hydrolysis product of sinigrin, which is the dominant glucosinolate in *B. nigra* (Gols *et al.*, 2009), cannot explain the observed wasp behavior, as no correlation was found between aphid density and the concentration of this compound in the plants' headspace. Such a finding would indicate that in *B. nigra*, allyl isothiocyanate does not play a primary role in the foraging behavior of this wasp species. Alternatively, it can be speculated that specialist aphids, such as *B. brassicae* manipulate plant volatile emission in *B. nigra* that is attractive to one of their important natural enemies, in order to reduce parasitoid recruitment, but this needs further research. However, while 100 aphids is a relatively mild infestation on a large plant such as *B. nigra*, the exponential population growth curve of aphids implies that at such a density the plant will rapidly become heavily infested by *B. brassicae* aphids. Thus, the higher aphid density used in this study may already be indicative to the parasitoid that host plant quality will probably deteriorate soon, with potential consequences for their offspring's fitness (Yoneya & Miki, 2015).

The emission of many of the plant volatile compounds responded nonlinearly to increasing aphid density, be this for the treatments with only aphids or for dually challenged plants. For many individual compounds, as well as for the overall volatile blend, plants infested with 25 or 100 aphids were more similar to each other than to plants infested with the intermediate density of 50 aphids, although the effects were more pronounced for single infestation with aphids than for the dually challenged treatments, and no methodological or functional explanations could be found for this. No blend characteristics could be linked to the observed behavior for any of the wasp species studied, and it is apparent that wasp behavior cannot simply be explained solely by quantitative differences in the overall emitted blends. What the observed behavior does suggest, however, is that in this system, *B. brassicae* aphid infestation, especially at higher densities, may suppress volatiles that are crucial for host-plant location, or alternatively, induce a volatile blend that is repellent. While analyzing the induced plant volatiles does not provide definitive answers in our case, it is known that insects perceive volatiles in concentrations much below the current detection thresholds, and they may be responding to very subtle differences in the volatile blend. Specific knowledge of the exact cues used by foraging wasps is limited and difficult to obtain, leading to difficulties in identifying blend characteristics that are key for successful foraging (Turlings & Fritzsche, 1999; Gols *et al.*, 2011). For *C. glomerata*, compounds that elicit an electroantennogram (EAG) response have been identified (Smid *et al.*, 2002). However, *B. nigra* does not emit any of these compounds,

and yet *C. glomerata* strongly responds to *B. nigra* volatiles with which they have no recent history (insects were reared from hosts on *B. oleracea* for several generations), indicating the involvement of other compounds. Identifying key blend features is an imposing task, given the many aspects of the volatile blend that may be important for wasp foraging. However, many features have proven to be important in foraging decisions, such as the ratios in which certain compounds are present, the presence of a given compound within a certain background, or the strong influence of minor blend constituents (Bruce & Pickett, 2011; Clavijo McCormick *et al.*, 2012; Clavijo McCormick *et al.*, 2014 and references within).

The task of deciphering the active components of plant volatile blends becomes infinitely more complicated when considering the complex foraging environments faced by parasitoid wasps, including changes in (non)host density. Variation in herbivore density is known to affect tritrophic interactions in the case of single herbivore attack (Dicke *et al.*, 1988; Gols *et al.*, 2003; Shiojiri *et al.*, 2010; Girling *et al.*, 2011), and also to affect induced plant volatile emissions, with the effects of density being either linear or nonlinear depending on the plant and insect species involved (Maeda & Takabayashi, 2001; Horiuchi *et al.*, 2003; Shiojiri *et al.*, 2010; Girling *et al.*, 2011; Truong *et al.*, 2014). For multiple attack scenarios, relatively little is known about the effects of herbivore density, although the effect of non-host herbivore density has been shown to trickle up to affect the third trophic level (Zhang *et al.*, 2009). Important lessons can be drawn from our results. We see that the plant volatile response to increasing aphid density is nonlinear, and if we had not included a third density, a linear volatile response could have been erroneously inferred, based on the behavioral data. This demonstrates the importance of having more than two densities in the comparison (see also Zhang *et al.*, 2009), with a greater risk of drawing incomplete conclusions when using two densities. Ideally, studying the relationship between herbivore density and volatile emissions should include an even wider range of herbivore densities in order to have a proper assessment of the kinetics of volatile induction. Our study also shows that the scaling up of the complexity in dual attack scenarios is not a straightforward procedure. We show that simple correlations between behavior and volatile emissions in pairwise comparisons may suggest the importance of certain volatiles in explaining behavioral attraction, whereas dose-response type analyses reveal that this approach may be too simple. Unfortunately, it has appeared to be very difficult to identify the exact blend that attracts parasitoids; such information is not available for any parasitoid species, despite 25 years of research into this (e.g. Turlings & Fritzsche, 1999; Gols *et al.*, 2011). Without understanding what specific cues are used by the wasp species being studied, it is difficult to exploit a

headspace data set to its fullest potential.

In conclusion, we have shown that even within the same study system, dual herbivore attack does not affect foraging decisions of each parasitoid species in the same way, and that aphid infestation, whether they be a parasitoid's host or a non-host, can have strong influence on foraging behavior, with these effects being density dependent. Foraging wasps use volatile cues from infested plants to locate their hosts. However, the strong dichotomy we observed between aphid effects on foraging behavior and the induced volatile blends illustrates that while we may have many clues, we are still a long way away from having a thorough understanding of the intricate processes underlying parasitoid foraging decisions in a tritrophic context.

Acknowledgments

We thank Léon Westerd, Frans van Aggelen, André Gidding and Joop Woelke for culturing the insects, and Wageningen University & Research Centre's experimental farm (Unifarm) for rearing the plants. This work, as part of the European Science Foundation EUROCORES Programme EuroVOL, was supported from funds by the Earth and Life Sciences Council (ALW) of the Netherlands Organisation for Scientific Research (NWO) and by the National Research Council of Italy. NWO is further acknowledged for funding to N.E.F. (NWO/ALW Veni grant no. 863.09.002).

Supporting information

Supporting information Table S1. Volatile emissions[a] by *Brassica nigra* plants from uninfested plants (C) and in response to *Pieris brassicae* egg infestation (E), *P. brassicae* caterpillar feeding (P), *Brevicoryne brassicae* aphids at each of the 3 densities (A25, A50, A100), dual infestation by aphids and caterpillars at each of the 3 densities (A25+P, A50+P, A100+P), and dual infestation by eggs and aphids at 2 densities (A50+E, A100+E)

Treatment →	C	E	P	A25	A50	A100	A25+P	A50+P	A100+P	A50+E	A100+E
Compound ↓	(N=10)	(N=10)	(N=8)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)
Ketones											
3-Pentanone	5.0 ±1.2	3.6 ±1.2	9±2	7±2	3.6 ±1.2	3.5 ±1.1	7.8 ±1.2	8.0 ±1.3	9.6 ±2.3	5.6 ±1.0	3.7 ±1.5
Alcohols											
1-Penten-3-ol	35±9	20 ±5	53 ±17	28±9	13±3	22 ±5	56 ±19	40 ±5	80 ±28	41 ±13	19 ±5
(*Z*)-3-Hexen-1-ol	37±15	18 ±6	29 ±3	19 ±4	18 ±7	19±4	56 ±15	40 ±7	38±7	26±8	12±3
DL-Menthol	55 ±16	36±10	49 ±17	58 ±25	50 ±15	47±17	31 ±9	37±12	29 ±13	23 ±4	46 ±12
Esters											
(*Z*)-3-Hexen-1-ol acetate	124±61	84±50	131±29	72 ±26	47 ±18	66 ±19	312 ±146	134 ±29	152 ±26	73 ±25	39 ±13
N -and/or S- containing											
2-Butenenitrile	1.1 ±0.6	0.8 ±0.6	2.2 ±0.5	1.9 ±0.8	0.35 ±0.25	1.2 ±0.3	4.5 ±1.9	2.8 ±0.8	3.2 ±0.9	0.4 ±0.3	1.1 ±0.4
3-Butenenitrile	9 ±3	5±3	19±4	7 ±3	4.1 ±1.4	11 ±6	25 ±8	22 ±4	23 ±4	5.6 ±1.5	6.4 ±1.2
2-Methylbutanenitrile	5.1 ±1.8	3±2	6.7 ±1.7	4.6 ±1.9	3.7 ±1.5	2.0 ±0.7	10 ±5	3.6 ±1.0	5.6 ±1.1	2.5 ±1.4	1.9 ±0.7
Dimethyl disulfide	41 ±16	32 ±11	26 ±12	25 ±6	13 ±3	53 ±37	21 ±5	34 ±7	16 ±5	16 ±6	20 ±4
Allyl isothiocyanate	196 ±102	37 ±11	294 ±99	25 ±5	50 ±25	331 ±217	357±127	263 ±112	326 ±114	110 ±45	54 ±14
Terpenoids											
Sabinene	6 ±2	6 ±3	3.4 ±1.5	4 ±2	3.9 ±1.5	3.3 ±1.0	3.5 ±1.6	4.6 ±1.5	2.7 ±0.7	5 ±3	4.2 ±1.2
Myrcene	5.9 ±1.3	6.2 ±1.4	6.7 ±1.4	7.1 ±1.5	7 ±2	6.2 ±0.9	7 ±2	6.8 ±0.9	5.5 ±0.6	5.1 ±1.0	7.2 ±1.2
(*E*)-DMNT[b]	30 ±7	69 ±40	192 ±63	38 ±16	31±10	31 ±8	167 ±59	132±64	38 ±12	35 ±17	53±34

Treatment →	C	E	P	A25	A50	A100	A25+P	A50+P	A100+P	A50+E	A100+E
Compound ↓	(N=10)	(N=10)	(N=8)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)
Menthone	14 ±6	7±2	8±3	11±6	9 ±3	10±5	5.7 ±0.8	8±4	8±5	5.8 ±1.0	12 ±4
Isomenthone	3.6 ±1.3	2.6 ±0.8	3.1 ±1.5	4 ±2	2.8 ±1.0	3.3 ±1.4	2.1 ±0.3	3.0 ±1.5	3.0 ±1.6	1.8 ±0.4	3.4 ±1.1
7-α-H-Silphiperfol-5-ene	47 ±25	63 ±28	136 ±44	56 ±18	57 ±24	31 ±6	43±16	60±20	37±15	90±23	84 ±44
Presilphiperfol-7-ene	12 ±9	6±3	19±10	13 ±5	7 ±3	6.1 ±1.7	7 ±2	6 ±3	6 ±4	14±4	12±7
7-β-H-Silphiperfol-5-ene	14 ±8	17±9	38±12	15±6	19 ±9	8.1 ±1.7	12 ±5	18 ±6	10 ±5	27±7	22 ±12
Silphiperfola-5,7(14)-diene	0.4 ±0.2	0.7 ±0.3	1.6 ±0.6	0.8 ±0.2	0.6 ±0.3	0.43 ±0.12	0.6 ±0.3	0.5 ±0.3	0.5 ±0.2	1.2 ±0.4	1.1 ±0.8
Silphiperfol-6-ene	7 ±4	8 ±4	19±7	7 ±3	10 ±5	3.9 ±0.8	6 ±3	10 ±4	5 ±2	13 ±4	14 ±9
α-Funebrene	10±5	2.4 ±1.2	9 ±3	6 ±2	2.7 ±0.9	2.9 ±0.7	5.4 ±1.9	6 ±2	8 ±2	7 ±3	9 ±8
Longifolene	4.6 ±0.5	4.2 ±0.5	4.1 ±0.8	3.4 ±0.4	4.9 ±0.7	3.8 ±0.5	4.5 ±0.9	4.2 ±0.5	3.5 ±0.3	4.5 ±0.5	4.1 ±0.5
7-epi-α-Cedrene	3.1 ±0.9	1.7 ±0.4	3.3 ±0.6	2.7 ±0.6	2.4 ±0.6	1.8 ±0.4	2.7 ±0.7	3.0 ±0.3	2.7 ±0.4	2.8 ±0.7	3.0 ±1.3
β-Caryophyllene	3.1 ±1.8	55 ±22	17±10	5±2	1.3 ±0.4	7 ±3	10 ±4	21±11	10 ±5	47 ±19	6 ±2
α-Ionone	0.1 ±0.1	0	0.3 ±0.3	0.29 ±0.15	1.4 ±0.8	1.0 ±0.3	0.5 ±0.3	0.7 ±0.4	0.3 ±0.3	0.1 ±0.1	0.6 ±0.3
α-Humulene	3 ±2	9 ±3	8 ±2	4.2 ±1.3	4 ±2	2.7 ±0.5	5.2 ±1.5	7 ±3	4.8 ±1.7	9±2	9 ±7
(*E*,*E*)-α-Farnesene	3.9 ±1.0	4.1 ±1.5	8±2	2.2 ±0.7	23 ±12	6 ±3	9 ±3	6 ±2	6 ±2	8 ±3	7 ±3
(*E*,*E*)-TMTT [c]	43 ±13	26 ±12	43 ±15	32 ±14	31 ±11	37±13	100 ±29	40±16	38±21	46 ±11	94±50
TUT [d]	2.3 ±1.2	2.1 ±0.9	9±4	2.1 ±0.9	2.2 ±1.2	1.5 ±0.4	2.8 ±1.9	2.9 ±1.6	1.8 ±0.7	4.9 ±1.6	9 ±7
Cembrene	5 ±2	3.0 ±1.1	3.2 ±1.3	1.3 ±07	2.9 ±1.3	2.9 ±1.4	3.6 ±1.3	1.4 ±0.7	1.5 ±1.3	2.1 ±0.6	2.3 ±1.0
Unknown											
Unknown compound 1	37 ±3	32±4	32±4	26 ±4	32 ±3	37 ±4	31 ±3	30 ±3	34 ±4	35 ±5	37 ±5
Unknown compound 2	7.6 ±0.5	6.6 ±0.9	7.9 ±1.1	5.6 ±1.0	6.7 ±0.8	7.2 ±0.6	7.5 ±0.6	6.8 ±0.6	7.1 ±0.8	7.6 ±0.8	7.6 ±0.7
Unknown compound 3	1.8 ±1.1	1.8 ±0.7	4.2 ±1.9	3.1 ±0.8	1.0 ±0.4	1.9 ±0.5	2.8 ±1.7	1.2 ±0.8	1.9 ±0.9	3.3 ±1.0	3 ±24

Treatment →	C	E	P	A25	A50	A100	A25+P	A50+P	A100+P	A50+E	A100+E
Compound ↓	(N=10)	(N=10)	(N=8)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)	(N=9)
Unknown compound 4	0.4 ±0.2	0.5 ±0.3	1.1 ±0.4	0.3 ±0.2	0.7 ±0.4	0.09 ±0.06	0.3 ±0.3	0.7 ±0.2	0.1 ±0.1	0.7 ±0.4	0.7 ±0.5
Unknown compound 5	4.8 ±0.9	4.1 ±0.9	3.3 ±1.0	2.2 ±0.5	3.4 ±0.8	3.6 ±0.7	4.3 ±1.8	4.8 ±1.1	3.1 ±0.9	3.3 ±0.7	4.0 ±0.8
Unknown compound 6	16.2 ±1.4	15 ±4	17 ±2	13 ±2	15 ±2	14.8 ±1.1	26.0 ±1.5	15.5 ±1.8	13.1 ±1.3	18 ±2	17.18 ±1.5
Unknown compound 7	2.0 ±0.7	1.7 ±0.5	3.4 ±1.4	2.0 ±0.6	2.9 ±1.0	2.9 ±0.5	2.8 ±0.7	4.0 ±1.5	3.0 ±0.7	1.9 ±0.5	3.0 ±1.0
Total	855 ±127	644 ±140	1276 ±168	562 ±94	539 ±62	840 ±304	1393 ±284	1051 ±164	983 ±134	754 ±93	687 ±110

[a] Volatile emissions are given as mean peak area ±SE/g fresh weight of foliage divided by 10^4 with the number of samples between brackets

[b] (*E*)-DMNT= (E)-4,8-Dimethylnona-1,3,7-triene

[c] (*E,E*)-TMTT= (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene

[d] TUT = Tricyclo[6.3.0.0(1,5)]undec-2-en-4-one, 2,3,5,9-tetramethyl

Chapter 5

Compatible and incompatible pathogen-plant interactions differentially affect plant volatile emissions and the attraction of parasitoid wasps

Camille Ponzio, Berhane T. Weldegergis, Marcel Dicke & Rieta Gols

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

Accepted for publication in Functional Ecology

ABSTRACT

The effects of multiple insect attacks on herbivore-induced plant volatiles and carnivorous arthropods are increasingly studied. Phytopathogens also represent an important threat to plants, and plant defense strategies against pathogens and insects are strongly interconnected, yet the potential impact of pathogens on insect-induced volatiles has been largely overlooked, and degree of pathogenicity rarely considered. We investigated how pathogen challenge, with virulent and avirulent strains of *Xanthomonas campestris* either alone or with simultaneous *Pieris brassicae* caterpillar herbivory, affected the volatile emissions of *Brassica nigra* plants. The impact of these volatiles on the foraging behavior of *Cotesia glomerata* parasitoids was then assessed. Pathogens themselves induced volatiles that were highly attractive to parasitoids, and enhanced the attractiveness of host-infested plant volatiles. Chemical analyses revealed that virulent and avirulent strains differentially induced plant volatiles, with primarily sesquiterpene, homoterpene and green leaf volatile compounds contributing to the differences. Strong similarities were found in the blends induced by the virulent strain and caterpillar herbivory. Challenge by either virulent or avirulent pathogens has a significant impact on plant chemistry and its interactions with other community members, demonstrating the importance of integrating pathogen- and insect-based research to broaden our knowledge of plant defenses under conditions of increasing complexity.

Keywords: bacterial pathogen, *Xanthomonas campestris*, *Pieris brassicae*, induced plant volatiles, parasitoid foraging, virulent, avirulent.

Introduction

The attack of a plant by deleterious organisms such as herbivores or pathogens activates plant defense responses, and sets off a cascade of events, which includes the production and emission of volatile organic compounds (Stout *et al.*, 2006; Howe & Jander, 2008). Herbivore-induced plant volatiles (HIPVs) are of strong ecological relevance, as they are exploited as foraging cues by the natural enemies of the attacking herbivores (Dicke & Baldwin, 2010), however studies of volatile-mediated interactions have largely focused on interactions involving a single insect herbivore and its associated natural enemy. There has been a shift towards studying multiple plant challengers (Dicke *et al.*, 2009; De Rijk *et al.*, 2013), yet work investigating the effects of multiple attack on tritrophic interactions has focused nearly exclusively on insect herbivores. However, in nature, plants are also frequently attacked by phytopathogens, often in conjunction with these insect herbivores.

Plant pathogens, by their omnipresence and diversity, represent a serious threat to plants, and their potential impact on arthropod-centered tritrophic interactions is not to be disregarded. While there are many studies investigating the direct effects of pathogen infection on insect herbivore performance and feeding preference (Stout *et al.*, 2006 and references within), the impact of pathogen-induced volatiles on plant-associated insect communities, particularly interactions with members of the higher trophic levels, has been largely disregarded. Few studies have tried to frame interactions between herbivorous attackers and plant pathogens in a wider ecological context, and examine how the effects affect other plant community members (reviewed by Tack & Dicke, 2013).

Characterizing pathogen-induced plant volatiles is not recent, and it is known that pathogen infection alone induces plant volatile emissions (Doughty *et al.*, 1996; Piel *et al.*, 1997; Jansen, 2011), and some of these volatiles have been shown to function as inhibitors of further pathogen colonization on the plant (Cardoza *et al.*, 2002). Pathogen-induced plant volatiles can also influence interactions between plants and herbivores by increasing the attractiveness of diseased plants to herbivores (Piesik *et al.*, 2011), or they can be used by ovipositing female insects to discriminate between healthy and infected plants (Dötterl *et al.*, 2009). With vector-transmitted plant diseases, induced volatiles also play an important role in mediating interactions between the pathogen and its insect vectors, influencing vector behavior to increase dissemination to other plants (McLeod *et al.*, 2005; Mauck *et al.*, 2010; Mann *et al.*, 2012), or even decreasing plant attractiveness to non-vector herbivores (van Molken *et al.*, 2012).

The effect of pathogens on herbivore-induced plant volatiles and subsequent volatile-

mediated foraging of the natural enemies of herbivores is largely unexplored territory. There has been a recent growing interest in this field, demonstrating how pathogen infection in combination with herbivory may affect the foraging behavior of parasitoid wasps (Hodge & Powell, 2008; De Oliveira *et al.*, 2014; Liu *et al.*, 2014; Martini *et al.*, 2014). Notably, in all cases the interactions studied are that of a pathogen, an insect vector and its parasitoid, with these being of great interest from a co-evolutionary perspective because of the tight link between pathogen and vector. However, little is known about the effects of pathogen infection in systems where pathogen and herbivore evolved independently from one another. There are few studies that have demonstrated that pathogen infection of a plant can affect the behavior of natural enemies of herbivores present on the plant at the same time (Cardoza *et al.*, 2003b; Rostás *et al.*, 2006; Tack *et al.*, 2012).

One commonality within the body of work on pathogen effects on tritrophic interactions is that in all cases, the plant pathogen is virulent on the tested plant species. However, the outcome of pathogen challenge can be broadly categorized into one of two possible outcomes: a compatible interaction (successful infection leading to disease), or an incompatible interaction (successful plant defense) (Glazebrook, 2005), though there are different degrees of susceptibility and resistance. During an incompatible interaction, plant resistance can trigger a hypersensitive response (HR), which is a programed cell death response at the site of pathogen entry, and which restricts further progression of the pathogen (Glazebrook, 2005). These two possible outcomes of pathogen challenge can directly, and differentially, affect herbivores feeding on the plant (Cui *et al.*, 2002). There is also evidence that compatible and incompatible plant-pathogen interactions can induce different volatile blends when a plant is challenged by either pathogen alone (Huang *et al.*, 2003), or when the pathogen is in combination with herbivory (Cardoza & Tumlinson, 2006). However, to date, the impact of these different plant-pathogen interactions, regarding pathogenicity, on natural enemies of herbivores has not been assessed.

The goal of this study was to investigate the effects of pathogen virulence on indirect plant defenses, that is, the promotion of natural enemy effectiveness against a subsequent herbivorous attacker. To achieve this, we asked if virulent and avirulent pathogen challenge would differentially affect the plant volatile blends they induce, and determined if these volatile blends affected tritrophic interactions. Previous work on the same system investigated in this study has shown that infection with the bacterium *Xanthomonas campestris* pv. *campestris* induces plant volatiles which in turn affect parasitoid foraging behavior (Ponzio *et al.*, 2014). We hypothesized that attack by an avirulent pathogen strain would result in the production of qualitatively and quantitatively different volatile blends from those induced by the virulent strain, which would differentially affect parasitoid foraging

behavior. To investigate this, we used a naturally occurring study system, comprising of the annual brassicaceous plant *Brassica nigra*, caterpillars of a common insect herbivore on brassicaceous plants, *Pieris brassicae*, and its associated parasitoid wasp, *Cotesia glomerata*. As microbial attackers, we selected the necrotrophic bacterial pathogen *Xanthomonas campestris*, and chose two related pathovars which both affect brassicaceous plant species, and which either cause disease on *B. nigra* (*X. campestris* pv. *campestris*) (compatible interaction), or induce a hypersensitive response (*X. campestris* pv. *incanae*) (incompatible interaction).

Material and methods

Plant and insect material

Brassica nigra plants were grown from seeds originating from a local population along the Rhine River in Wageningen (the Netherlands). Three-to-four-week-old plants were used in all experiments. All plant and insect cultures were maintained in a greenhouse at 22 ± 2 °C, 60–70% relative humidity (r.h.), and 16:8 light:dark regime. Caterpillars of *P. brassicae* were reared on Brussels sprout plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus), with the parasitoid wasp *C. glomerata* reared on *P. brassicae*. Cocoons of *C. glomerata* were placed in a 30 × 30 × 30 cm cage (Bugdorm, Taichung, Taiwan) and supplied with a 6% sucrose solution and honey. The cage was kept in a climate-controlled cabinet at 21 ± 1 °C and a light-dark regime of 16:8. Mated, three-to-seven-day-old adult female wasps were used in the wind tunnel bioassays. Individual wasps were used only once and were considered naïve (i.e. they had no previous experience with hosts, host products or plants).

Bacteria preparation

The two *Xanthomonas campestris* pathovars were selected based on their (in)ability to infect *B. nigra* plants. Both bacterial pathovars were kept at -80 °C in a 50% glycerol solution. *X. campestris* pv. *campestris* was obtained from the laboratory of Plant-Microbe Interactions of Utrecht University in the Netherlands and was virulent on *B. nigra*, i.e. the plants developed disease symptoms. *X. campestris* pv. *incanae* was obtained from the French Collection of Plant Pathogenic Bacteria (CFBP) in Angers, France, and was avirulent, leading to a hypersensitive response at the site of infiltration. Fresh inoculum was obtained by culturing 250 µL of stock solution in Erlenmeyer flasks containing 30 ml of Difco Nutrient Broth (Becton, Dickinson and Company, Sparks, MD, USA), and placed in a shaker at 28 °C and 170 r.p.m. for 18-24h. The broth was then transferred to a 50 mL tube, and centrifuged at 3000× *g* for 10 min. The bacterial cells were re-suspended in a 10 mM $MgSO_4$ buffer

solution and the OD600 was adjusted to 0.066 (approximately 1 x 10^8 cfu/ml).

Plant treatments

B. nigra plants were inoculated with the bacterial suspension by infiltration, using a needleless syringe, on the abaxial side of the largest fully developed leaf. Ten 4-to-5-mm-diameter spots were infiltrated per leaf. Leaves of control and caterpillar-infested plants were infiltrated with buffer only. After infiltration, all plants were placed in 35 × 35 × 60 cm mesh cages (Vermandel, Hulst, The Netherlands), with one treatment per cage, in a greenhouse kept at 25±2 °C, 70% r.h. All cages were covered with transparent plastic for the first 48h after infiltration to ensure sufficiently high humidity for successful infection. After 48h, the dual infestation or herbivore-only treatments were infested with 20 newly hatched *P. brassicae* caterpillars on the leaf immediately above the infiltrated leaf. Plants were used in wind tunnel assays and headspace collection (see below) after 24h of caterpillar feeding without removing the caterpillars. All plants were tested 72h post-infiltration. Six treatments were compared in wind tunnel bioassays and headspace analyses: (1) healthy control plants, (2) plants infested by *P. brassicae* caterpillars, (3) and (4) plants infected with either *X. campestris* pv. *campestris* or *X. campestris* pv. *incanae*, (5) and (6) plants dually challenged with *P. brassicae* caterpillars and either *X. campestris* pv. *campestris* or *X. campestris* pv. *incanae*.

Wind tunnel assays

Two-choice behavioral bioassays were done in a wind tunnel at 25±2 °C, 60% r.h. and a wind speed of 10 cm/s, as previously described by Geervliet *et al.* (1994). Plants infected with either pathogen pathovar (treatments 3 and 4) or infested with caterpillars (treatment 2) were tested against healthy plants (treatment 1). All of the pathogen-infection treatments, either pathogen-only (treatments 3 and 4) or in combination with caterpillars (treatments 5 and 6), were also tested against caterpillar-infested plants (treatment 2). When possible, the same treatment pair was tested for both pathovars on the same day to eliminate day effects. The bioassays and wasp handling were carried out in a similar manner as in Ponzio *et al.* (2014). Females were released in two to three groups of five individuals from a release platform downwind of the plants, and the results were subsequently pooled and considered a replicate for the statistical analysis. The observation period was set to 15 minutes, and non-responding wasps were scored and subsequently excluded from statistical analyses. The position of the plants was exchanged after each released group of females to exclude any potential positional bias. Eight to ten replicates were tested for each treatment combination. Each pair of tested plants was considered a replicate, with

10 to 15 wasps released per replicate.

Collection and analysis of plant headspace volatiles

Plant volatiles were collected for each of the six treatments listed previously, from separate sets of plants than those used for the behavioral assays, with 10 to 12 plants sampled per treatment, and caterpillars remaining on the herbivore treated plants. With the *X. campestris* pv. *campestris* infection treatments, disease severity was scored based on the extent of yellowing and necrosis ('low': symptoms limited to tissue immediately around the infiltration site; 'medium': symptoms present on up to a third of the leaf; 'high': over a third of the leaf is affected, Fig. 1). In order to exclude any contribution from the sampling set-up, air from empty jars was sampled at the start and end of the experiment, as well as from soil-filled pots wrapped in aluminum foil. Headspace collection and the subsequent analysis of the volatiles via GC-MS were carried out as described in detail by Ponzio *et al.* (2016). The pots were wrapped in aluminum foil to minimize the effect of soil and pot-derived volatiles, and the plants were placed in the glass vessels 30 min before trapping began. Headspace collection was done for two hours at a flow rate of 300 ml/min, always at the same time of day, and no treatment was trapped more than once per day.

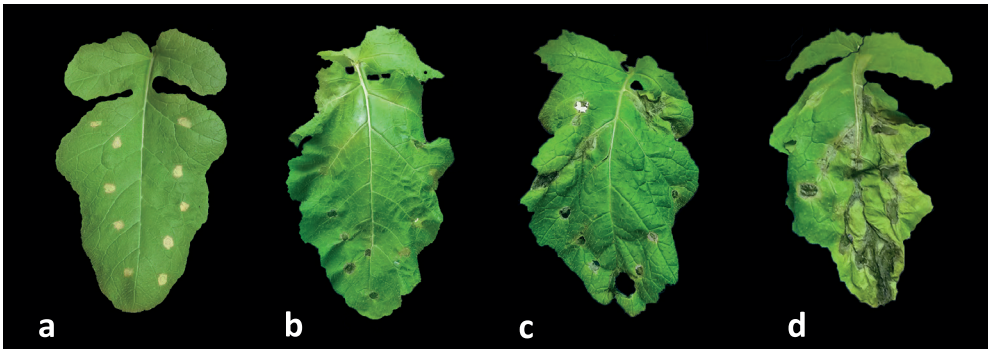


Figure 1. Symptoms development on *B. nigra* plants 72 hours after infiltration of a bacterial plant pathogen. Hypersensitive response caused by the avirulent *Xanthomonas campestris* pv. *incanae* strain (a), necrosis and yellowing caused by the virulent *Xanthomonas campestris* pv. *campestris* in the case of low (b), medium (c), or high (d) symptom severity.

Statistical analysis

To investigate whether parasitoid preferences differed when various combinations of plant treatments were offered, the data were analyzed using logistic regression in SAS version 9.2 (SAS Institute Inc. Cary, NC, USA) with plant treatment as a fixed factor. In

case of overdispersion, correction was made for this by allowing the variance functions of the binomial distribution to have a multiplicative overdispersion factor. In the comparison with control plants, the number of wasps choosing the attacker-infested plants out of the total number of responding wasps was entered as the response variable. In the analysis of dual versus single attack, the number of wasps choosing the dually infested plant out of the total number of responding wasps was entered as the response variable. Each bioassay with one pair of plants and 10 to 15 released wasps served as a replicate. To determine within each comparison whether there was a significant preference for one of the offered plant treatments, we tested H_0: logit = 0, which equals testing equal preference or p = 0.5.

The volatile emission patterns, quantified as peak areas divided by the fresh mass of the aerial portion of the plant, were analyzed through multivariate data analysis using OPLS-DA (orthogonal projection to latent structures discriminant analysis) (Eriksson *et al.*, 2006). This projection method determines whether samples belonging to the different treatment groups can be separated based on quantitative and qualitative differences in their volatile blends. To do this, a Y-data matrix of dummy variables is included, assigning a sample to its respective treatment group. The OPLS-DA extension in the SIMCA-14 software program (Umetrics AB, Umeå, Sweden) then computes the variation of X which is predictive (correlation between X and Y) and the part of the variation which is orthogonal (unrelated) to Y (Trygg & Wold, 2002). Removal of non-correlated variation from the model enhances its interpretability. The results of the analysis are visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components and the relationships among the variables. The analysis additionally shows the variable importance in the projection (VIP) of each variable (the different compounds), with variables having VIP values greater than 1 being most influential in the model (Eriksson *et al.*, 2006). Data were log-transformed, mean-centered and scaled to unit variance before they were subjected to the analysis.

Results

Wasp foraging behavior

Foraging behavior of the parasitoid wasps was strongly affected by pathogen infection of the plants (Fig. 2). When wasps had the choice between healthy plants and plants challenged by either caterpillars, virulent *X. campestris* pv. *campestris* or avirulent, HR-inducing *X. campestris* pv. *incanae*, they were always significantly more attracted to challenged than to control plants. However, the strength of the attraction was different between treatment

pairs (GLM, df = 2, χ^2 = 21.02, P < 0.001), from most to least attractive: caterpillar-infested, virulent pathovar, and avirulent pathovar challenged plants (Fig. 2, top panel). When wasps were offered a choice between volatiles of host-infested plants and plants dually challenged by hosts and either pathogen, they preferred the dually challenged plants and the strength of preference was similar regardless of the pathogen strain (GLM, df = 1, χ^2 = 0.86, P = 0.35, Fig. 2, middle panel). In a final set of comparisons, host-infested plants were tested against plants infected only with either pathogen to determine if the attractiveness of the pathogen-induced volatiles was strong enough to interfere with parasitoid foraging. Here, the wasps always preferred volatiles of the host-infested plants; however the strength of the preference was significantly affected by pathogen identity (GLM, df = 1, χ^2 = 6.59, P = 0.01), with volatile preference more strongly affected by the virulent pathovar than by the avirulent pathovar (Fig. 2, lower panel).

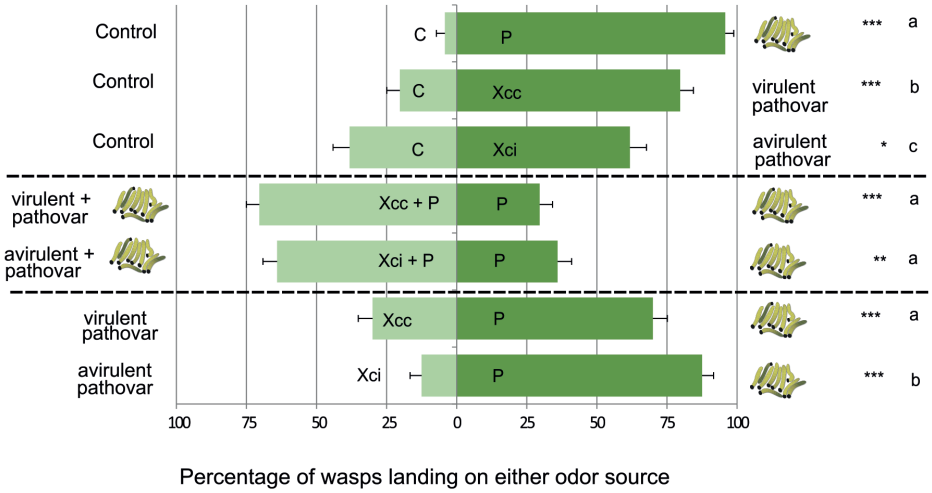


Figure 2. Preference of *C. glomerata* wasps in a two-choice setup in a wind tunnel (percentage ±SE), where plants were healthy controls plants (C), challenged with the virulent *Xanthomonas c*ampestris pv. *campestris* bacterium strain (Xcc) or with the avirulent *X. campestris* pv. *incanae* strain (Xci), infested with 20 first instar *P. brassicae* caterpillars (P), simultaneously challenged with the virulent strain and *P. brassicae* caterpillars (Xcc+P) or simultaneously challenged with the avirulent strain and *P. brassicae* caterpillars (Xci+P). Significant differences between treatments (P < 0.05) are indicated with different letters, with the treatment pairs analyzed in groups that are separated by the dashed lines. Asterisks indicate a preference which is significantly different from a 50:50 distribution within a choice test : * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

Plant volatiles

Analysis of the plants' headspace showed that the same 36 compounds were produced by plants of all treatments (Table 1). Differences between treatments are therefore due to quantitative differences in emission rates of the different compounds.

Table 1. Volatile emissions[a] by *Brassica nigra* plants from uninfested plants (C) and in response to virulent *Xanthomonas campestris* pv. *campestris* (Xcc), avirulent *Xanthomonas campestris* pv. *incanae* (Xci), *Pieris brassicae* caterpillar feeding (P), .*Xanthomonas campestris* pv. *campestris* with caterpillars (Xcc+P), and *Xanthomonas campestris* pv. *incanae* with caterpillars (Xci+P).

ID[b]	Treatment →	C	Xcc	Xci	P	Xcc+P	Xci+P
	Compound ↓	N= 9	N= 11	N= 10	N= 10	N= 12	N= 11
Ketones							
4	2-Pentanone	9.6±1.2	10.1±1.9	7.8±0.8	9.4±1.1	9.6±1.1	8±1.0
5	3-Pentanone	5.1±0.8	27±4	11.2±1.9	12±2	20±3	15±3
10	2-Methyl-2-cyclopenten-1-one	4.4±0.6	6.6±1.2	4.7±1.0	6.4±1.3	7±2	4.2±0.6
Alcohols							
3	1-Penten-3-ol	85±19	614±241	160±44	232±99	397±71	166±35
8	(*Z*)-3-Hexen-1-ol	15±5	121±32	35±11	36±10	78±18	31±10
17	3,4-Dimethylcyclohexanol	0.5±0.3	15±9	1.5±0.5	1.5±0.6	15±7	1.7±0.5
Aldehydes							
7	(*E*)-2-Hexenal	4±2	12±4	6±3	10±5	15±8	6±3
Esters							
14	(*Z*)-3-Hexen-1-ol, acetate	25±6	168±120	105±52	45±10	67±18	55±12
24	α -Terpinyl acetate	0.9±0.2	1.4±0.6	1.1±0.3	1.4±0.3	2.0±0.7	1.0±0.2
N and/or S containing compounds							
1	2-Butenenitrile	1±0.3	10±7	0.9±0.2	16±4	23±8	7±2
2	3-Butenenitrile	1.5±0.5	17±7	2.1±0.9	59±17	97±38	25±5
6	2-Methylbutanenitrile	57±48	41±32	16±12	82±72	28±14	19±5
9	Allyl isothiocyanate	50±22	3049±2864	80±22	265±77	327±109	166±58
Monoterpenes							
11	α-Pinene	33±5	39±8	38±6	42±6	35±6	37±6
12	Camphene	2.2±0.3	4.4±1.4	2.7±0.4	3.4±0.7	3.3±1.8	2.6±0.4
13	β-Myrcene	9.7±2.1	12±2	11±2	13±2	11±2	11±2
15	3-Carene	20±4	26±5	23±4	25±4	24±5	23±4
16	Tetrahydrolinalool	44±8	136±78	46±14	44±19	108±70	54±15
19	Menthone	8±5	3.1±0.6	7±5	3.1±0.5	8±6	4±2
20	Menthol	42±13	32±5	38±12	30±4	43±21	34±8
Homoterpenes							
18	(*E*)-DMNT[c]	3.0±1.7	22±8	6±3	108±59	71±24	32±5
34	(*E,E*)-TMTT[d]	7±4	25±12	6±4	16±5	42±17	15±5
Sesquiterpenes							
21	7- α -H-Silphiperfol-5-ene	15±7	22±15	37±15	23±12	31±13	44±18
22	Presilphiperfol-7-ene	2.1±0.9	3±3	4±2	2.7±1.8	2.5±1.3	2.7±1.2

ID [b]	Treatment →	C	Xcc	Xci	P	Xcc+P	Xci+P
	Compound ↓	N= 9	N= 11	N= 10	N= 10	N= 12	N= 11
23	7-β-H-Silphiperfol-5-ene	5±2	8±5	12±5	7±4	12±6	17±8
25	Silphiperfola-5,7(14)-diene	0.16±0.07	0.2±0.1	0.7±0.3	0.2±0.1	0.5±0.2	0.5±0.2
26	Silphiperfol-6-ene	2.6±1.1	4±2	5±2	4±2	6±3	9±4
27	α -Funebrene	4.2±1.5	4±2	10±6	7±4	6±3	7±3
28	Longifolene	5.8±0.7	6.6±0.7	5.3±0.9	7.0±0.8	5.4±0.6	6.7±0.8
29	β-Caryophyllene	4.8±1.9	3.2±1.5	8±3	19±8	7±3	5.5±1.3
30	(*E*)- β -Farnesene	2.1±0.7	2.7±0.8	8±6	3.01±1.1	3.4±0.9	12±9
31	(*E,E*)- α -Farnesene	106±34	148±36	78±22	162±46	186±49	75±16
35	TUTM [e]	2.3±1.5	2.0±1.3	4±3	4±3	2.3±1.4	6±2
36	IPDMOHM [f]	55±12	92±25	36±6	52±11	52±17	48±10
Unknown							
32	Unknown compound	275±90	474±134	333±92	461±112	342±95	340±39
33	Unknown compound	10.8±1.8	15±3	9.3±1.8	13±2	11.2±1.8	9.3±0.8
37	Total	863±130	5085±3374	1123±188	1773±275	2045±378	1250±101

[a] Volatile emissions are given as mean peak area ±SE/g fresh weight of foliage divided by 10^4 .

[b] ID corresponds with the numbers presented in Fig. 3b, Fig. 4b and 5b.

[c] (*E*)-DMNT= *(E)*-4,8-Dimethylnona-1,3,7-triene

[d] (*E,E*)-TMTT= 4,8,12-Trimethyl-1,3,7,11-tridecatetraene

[e] TUTM= Tricyclo[6.3.0.0(1,5)]undec-2-en-4-one, 2,3,5,9-tetramethyl-

[f] IPDMOHM = (7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol

An OPLS-DA model including all treatments resulted in a model with two predictive principal components (PC) (Fig. 3a). The first PC separated the control and avirulent pathovar treatments from the other treatments, while the second PC separated treatments that were singly and dually infested with *P. brassicae* from the treatments that did not have herbivory. Twelve compounds had a high discriminatory power, i.e. having a VIP value greater than 1, and all were associated with the three treatments that included *P. brassicae* (single and dual attack), and virulent pathovar challenge alone, listed by decreasing order of VIP value (and with corresponding ID number): (1) 2-butenenitrile, (3) 1-penten-3-ol, (5) 3-pentanone, (18) (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT), (2) 3-butenenitrile, (8) (*Z*)-3-hexen-1-ol, (17) 3,4-dimethylcyclohexanol, (9) allyl isothiocyanate, (6) 2-methylbutanenitrile, (31) (*E,E*)-α-farnesene, (7) (*E*)-2-hexenal, and an unknown compound (33) (Fig 3b).

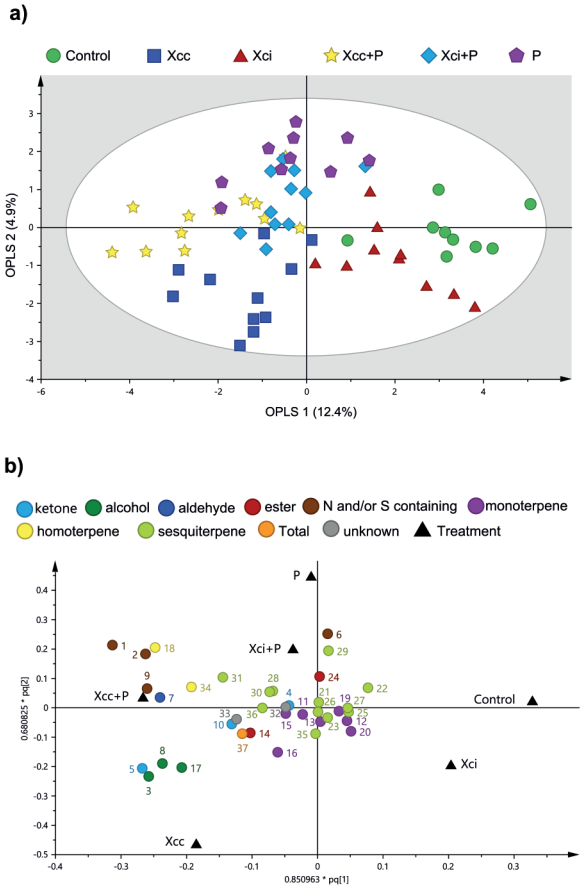


Figure 3. OPLS-DA comparison of the volatile blends emitted by individual *B. nigra* plants sampled after 72 h. **(a)** Score plot of the samples, with the percentage of explained variation in parentheses. Plants were healthy controls plants (C), challenged with the virulent *Xanthomonas campestris* pv. *campestris* strain (Xcc) or with the avirulent *X. campestris* pv. *incanae* strain (Xci), infested with 20 first instar *P. brassicae* caterpillars (P), simultaneously challenged with the virulent strain and caterpillars (Xcc+P) or simultaneously challenged with the avirulent strain and caterpillars (Xci+P). The OPLS-DA resulted in a model with two predictive components and two components orthogonal in X: R^2X=0.416 R^2Y=0.307 Q^2=0.141. The ellipse defines the Hotelling's T^2 confidence region (95%). **(b)** Loading plot of the two components of the OPLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 1.

To gain more insight into the effects of pathogen challenge on the volatile profiles, the three single attacker treatments - i.e. challenge by *P. brassicae*, the virulent and avirulent pathovars - were further analyzed in a separate model using OPLS-DA (Fig.4a). Here again, there were two significant PCs. The first separated avirulent pathovar-treated plants from the other two treatments, indicating a strong similarity between the plant volatiles induced by caterpillar herbivory and infection with the virulent pathovar. The second PC separated *P. brassicae*-infested plants from pathogen-challenged plants, which shows that pathogen-induced and herbivore-induced volatile blends are also specific to the organism type. Thirteen compounds had a VIP value greater than 1, and strongly contributed to separation between the treatments. Two of these, (6) 2-methylbutanenitrile and (22) presilphiperfol-7-ene, were more present in the avirulent pathovar treatment,

while (in decreasing order of VIP values, with corresponding ID code) (3) 1-penten-3-ol, (1) 2-butenenitrile, (2) 3-butenenitrile, (18) (*E*)-DMNT, (5) 3-pentanone, (17) 3,4-dimethylcyclohexanol, (8) (*Z*)-3-hexen-1-ol, (33) an unknown compound, (7) (*E*)-2-hexenal, (31) (*E,E*)-α-farnesene, and (9) allyl isothiocyanate were associated with *P. brassicae* herbivory or challenge with the virulent *X. campestris* pv. *campestris* (Fig. 4b). The three *P. brassicae* infestation treatments (*P. brassicae, P. brassicae* plus the virulent pathovar, and *P. brassicae* plus the avirulent pathovar) were also analyzed separately (not shown), however this model did not result in any significant PCs, indicating that the volatile blends induced by these treatments are highly similar even when plants are dually challenged with *P. brassicae* plus a pathogen.

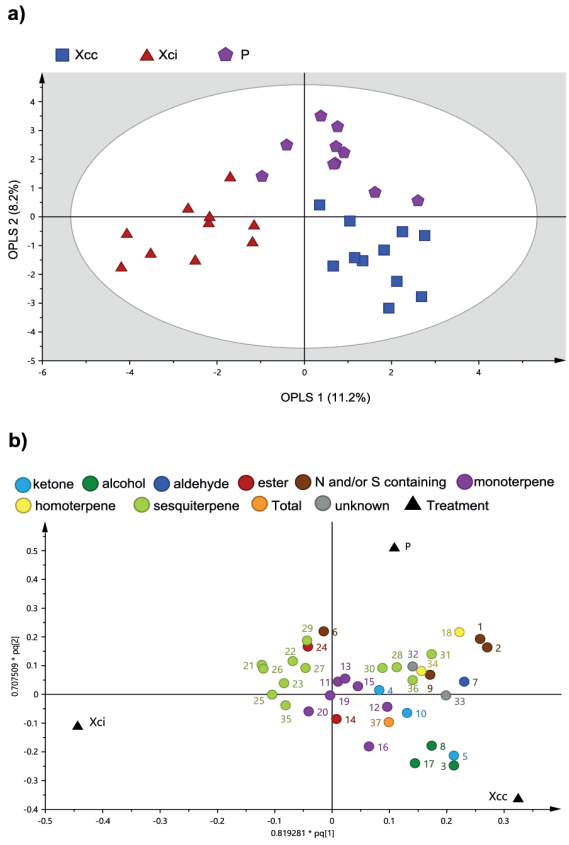


Figure 4. OPLS-DA comparison of the volatile compounds emitted by individual *B. nigra* plants sampled after 72 h, after challenge by a single attacker. **(a)** Score plot of the samples, with the percentage of explained variation in parentheses. Plants were challenged with the virulent *Xanthomonas campestris* pv. *campestris* bacterium strain (Xcc), with the avirulent *X. campestris* pv. *incanae* strain (Xci) or infested with 20 first instar *P. brassicae* caterpillars (P). The OPLS-DA resulted in a model with two predictive components and two components orthogonal in X: $R^2X=0.516$ $R^2Y=0.744$ $Q^2=0.472$. The ellipse defines the Hotelling's T^2 confidence region (95%). **(b)** Loading plot of the two components of the OPLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 1.

For the virulent pathovar, volatile profiles were further explored in relation to the severity of the symptoms, for both the treatments singly or dually challenged with *X. campestris* pv. *campestris*. The volatile blends of these plants were affected in a disease-severity dependent matter, irrespective of caterpillar presence on the plants. The OPLS-DA yielded a model

with two significant PCs, separating 'low' and 'medium' symptoms groups from the 'high' symptom group, and then further separating 'low' from 'medium' (Fig. 5a). Eighteen compounds were highly influential for the separation, with, in decreasing order of VIP value (and corresponding ID code noted), (26) silphiperfol-6-ene, (23) 7-β-H-silphiperfol-5-ene, (22) presilphiperfol-7-ene, (21) 7-α-H-silphiperfol-5-ene, and (25) silphiperfola-5,7(14)-diene being most abundant in the 'low' and 'medium' symptom classes, while (17) 3,4-dimethylcyclohexanol, (33) an unknown compound, (35) tricyclo[6.3.0.0(1,5)] undec-2-en-4-one,2,3,5,9-tetramethyl (TUTM), (3) 1-penten-3-ol, (9) allyl isothiocyanate, (10) 2-methyl-2-cyclopenten-1-one, (37) total emitted volatiles, (36) (7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol (IPDMOHM), (7) (*E*)-2-hexenal, (20) menthol, (16) tetrahydrolinalool, (2) 3-butenenitrile, and (5) 3-pentanone were more characteristic of the headspace of 'high'-symptom plants (Fig. 5b).

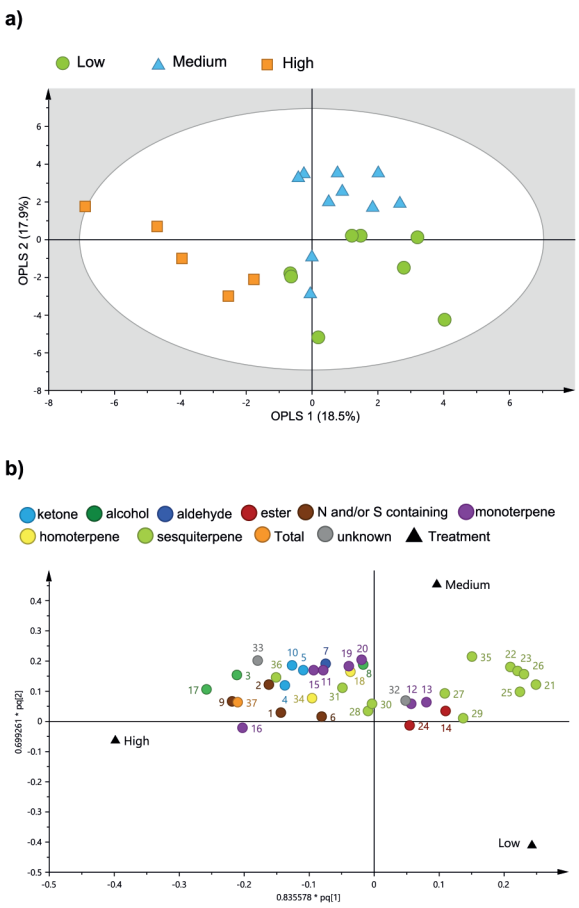


Figure 5. OPLS-DA comparison of the volatile compounds emitted by individual *B. nigra* plants sampled after 72 h, after challenge by the virulent pathogen strain, either alone or in combination with caterpillar herbivory. **(a)** Score plot of the samples, with the percentage of explained variation in parentheses. Plants were grouped according to the severity of the disease symptoms, irrespective of their treatment group, and were considered to have either low, medium, or high disease severity. The OPLS-DA resulted in a model with two predictive components: R^2X=0.364 R^2Y=0.534 Q^2=0.163. The ellipse defines the Hotelling's T^2 confidence region (95%). **(b)** Loading plot of the two components of the OPLS-DA, showing the contribution of each of the compounds towards the model. Numbers refer to the volatile compounds listed in Table 1.

Discussion

Volatile-mediated foraging behavior of parasitoids has been extensively studied, but has in large part been limited to plant-herbivore systems, despite the strong prevalence of plant pathogenic microbes in natural systems and their known effects on the induction of plant volatiles (Tack & Dicke, 2013). When pathogens are included in studies, they are limited to disease-causing strains, effectively overlooking the potential impact of unsuccessful pathogen infection. We combined attack by these two major plant threats in one study system, and showed that virulent and avirulent pathovars of the same phytopathogenic bacterial species induce quantitatively distinctive volatile blends that are both highly attractive to parasitoids, although caterpillar-infested plants remain most attractive. During dual attack, the wasps preferred the volatiles from dually challenged plants to those from plants infested with hosts alone. These results are in large part reflected in the composition of the headspace of the pathogen and/or herbivore treated plants. Furthermore, differences in pathogen-induced plant volatiles are correlated with disease severity and the chemical data indicate that the similarity of caterpillar-induced and virulent pathogen-induced volatile blends increases with disease severity.

When comparing the headspace of the three single attacker treatments, each induced its own unique volatile blend, but there was a high degree of similarity in the blends produced in response to herbivory and virulent pathogen challenge. Wasp foraging behavior was correlated to this; wasps exhibited a stronger preference for volatiles from plants induced by either *P. brassicae* or the virulent strain, compared to volatiles from plants induced by the avirulent pathovar. The virulent pathovar, *X. campestris* pv. *campestris*, induced a volatile blend which was quantitatively and qualitatively strikingly similar to the one induced by *P. brassicae* herbivory, which includes compound groups such as homoterpenes, green leaf volatiles (GLVs) and glucosinolate breakdown products, many of which are typically associated to caterpillar herbivory (Pare & Tumlinson, 1999). On the other hand, plants challenged by the avirulent, HR-inducing *X. campestris* pv. *incanae* produced a volatile blend that was quantitatively very different from both of these, and was primarily characterized by higher quantities of a subgroup of sesquiterpenes. GLVs and glucosinolate breakdown products are produced in response to tissue damage (Halkier & Gershenzon, 2006; Shiojiri *et al.*, 2006), and given the strong similarity between volatile blends induced by *P. brassicae* and the virulent pathovar, these results suggest that pathogen infection and caterpillar herbivory lead to similar damage responses by the plant. Such specificity in the induced volatile blend due to virulence or avirulence of pathogen strains has also been shown for the model pathogen *Pseudomonas syringae* (Croft

et al., 1993; Huang *et al.*, 2003; Huang *et al.*, 2005; Cardoza & Tumlinson, 2006). However, when plants simultaneously experience *P. brassicae* herbivory and pathogen infection, the pathogen specificity in the volatiles disappears, and all caterpillar-infested treatments have similar blend characteristics. Thus, caterpillar infestation not only overrides differences in volatile induction by the two pathovars but also the effects of pathogen induction per se.

Variation in wasp foraging behavior could be correlated to the chemical data in several instances. Although the two pathovars induce volatile blends with very different overall characteristics, remarkably, both induced volatile blends were highly attractive to wasps. This was also the case even in the complete absence of hosts, though the strength of the preference differed. These results suggest that the volatile components important for foraging by *C. glomerata* are also induced in response to pathogen infection, and that differences in parasitoid behavioral response are likely based on quantitative differences, as all treatments induced the same compounds. Interestingly, when plants were challenged by pathogen infection and herbivory simultaneously, their volatile blends were more attractive to *C. glomerata* than those emitted by plants only infested by caterpillars, although the volatile blends emitted by these three treatment groups could not be statistically separated based on the recorded chemical composition. Thus, there appears to be more subtle blend characteristics that influence its attractiveness to *C. glomerata*. This may include compounds emitted in quantities below the detection threshold. However, the apparent differences between the blends when challenged by the two pathovars alone were eliminated when plants are challenged by both caterpillars and bacteria. The few similar studies that have focused on related questions also did not find evidence for negative effects of pathogen infection on parasitoid foraging. In two cases parasitoid attraction or parasitism rate was enhanced when herbivore-infested plants were also infected with a fungus (Cardoza *et al.*, 2003b; Tack *et al.*, 2012) while in a third study, simultaneous herbivory and infection did not affect parasitoid preference, and plants infected only with the fungus were not attractive to wasps (Rostás *et al.*, 2006).

While the avirulent *X. campestris* pv. *incanae* consistently led to HR at the infiltration sites, we observed strong variation in the severity of the symptoms induced by the virulent *X. campestris* pv. *campestris*. As disease severity increased, the emitted volatiles showed increasing similarity with the caterpillar-induced blend, in terms of the most important compounds in characterizing the blends. Interestingly, the isomers and derivatives of silphiperfolene sesquiterpenes, which are important in separating 'low' and 'medium' diseased plants from the highly symptomatic plants, contribute in a similar way to the separation between the virulent and avirulent pathovars. This indicates that this group of compounds, or the biosynthetic pathways they stem from, may be important in

mounting a defense response against *X. campestris*. These compounds may be related to defense responses in *B. nigra*, as they are also strongly induced by *P. brassicae* eggs, more specifically by eggs which induced a hypersensitive response in the plant (Fatouros *et al.*, 2012). Sesquiterpenes in a broader sense have also been found to be more strongly induced in an incompatible interaction with a different *X. campestris* pathovar, *X. campestris* pv. *vesicatoria*, on pepper plants compared to a compatible interaction (Cardoza & Tumlinson, 2006), and also with *P. syringae* on tobacco (Huang *et al.*, 2003), or downy mildew on grape plants (Algarra Alarcon *et al.*, 2015).

It is intriguing that pathogen-challenged plants prove so attractive to parasitoid wasps, most notably in the absence of caterpillar hosts. Foraging wasps, especially when naive, will be highly inefficient when foraging on these plants. When addressing questions such as this, a common approach is to seek a functional explanation to volatile production, i.e. to what benefit would a plant emit volatiles that carnivorous arthropods would misinterpret. While herbivory does indeed elicit a volatile response which can then serve as cues for foraging natural enemies, volatile emissions can be induced by a multitude of stimuli, from abiotic and other biotic stresses that include pathogen infection, and also by exogenous application of phytohormones to plants (Bruinsma *et al.*, 2009; Holopainen & Gershenzon, 2010). All these stressors induce volatile induction via the same general pathways, though there is a degree of attacker specificity in the phytohormonal 'signal signature' (De Vos *et al.*, 2005; Tumlinson & Engelberth, 2008). Phytopathogens can also induce the emission of volatile compounds which are typically and traditionally associated with herbivory, such as GLVs, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene ((*E,E*)-TMTT) (Shiojiri *et al.*, 2006; Attaran *et al.*, 2008; Scala *et al.*, 2013) or (*E*)-DMNT (Sohrabi *et al.*, 2015), all of which show that these volatile compounds can also have important roles in direct plant defense against pathogens. We showed that *B. nigra* plants emit high levels of GLVs (Fig 4b), particularly (*Z*)-3-hexen-1-ol, when challenged with the virulent pathovar. These GLVs have been shown to be key cues for foraging *C. glomerata* wasps as demonstrated by Shiojiri *et al.* (2006), and supported by electroantennogram studies that previously demonstrated the importance of these volatiles for this wasp species (Smid *et al.*, 2002). While this may provide an explanation for attractiveness of the virulent pathovar, it may not be so easily applied to the avirulent pathovar, where GLV emissions were not so highly induced.

The similar effects of *X. campestris* pv. *campestris* infection and *P. brassicae* herbivory may in part be due to commonalities in the defense signaling pathways they induce. Defense against caterpillar herbivory is primarily mediated via induction of the jasmonic acid (JA) signaling pathway (Kessler & Baldwin, 2002; De Vos *et al.*, 2005). Involvement of this

pathway has also been shown for defense against *X. campestris* (Thaler *et al.*, 2004; De Vos *et al.*, 2006), though in this case defense was mediated by activation of the three main phytohormonal signaling pathways: salicylic acid (SA), JA and ethylene (ET), as shown for *X. campestris* pv. *armoraciae* in Arabidopsis (Ton *et al.*, 2002). In our study, induction of JA-dependent defenses by *X. campestris* and *P. brassicae* may lead to the production of similar volatile blends, with the extensive tissue damage caused by the virulent pathovar further increasing the similarity. Double induction of this pathway in the dually treated plants may have had an additive or synergistic effect on plant volatile responses, leading to the observed enhanced attractiveness of these dually challenged plants.

In conclusion, we show that pathogen challenge, whether or not it results in disease, can have profound effects on the emission of plant volatiles, and affect the behavior of parasitoid wasps that exploit these cues to locate their hosts. Such a strong initial attraction to pathogen-challenged plants is not in the parasitoid's best interest, as valuable time and energy will be spent foraging on host-free plants. However, *C. glomerata* has a wide dietary breadth in terms of plant species on which their caterpillar hosts are found, and, when naïve, they seem to respond to more general volatile cues to locate host-infested plants (Ponzio *et al.*, 2014). Parasitoid behavior can also be adaptive; despite the high attractiveness of pathogen-challenged plants, plants with hosts, or challenged by hosts and pathogen are more strongly preferred than plants challenged solely with the pathogen. So while a wasp may initially land and search for hosts on pathogen-challenged plants, it may gain a negative experience and then quickly move on to other, host-infested plants. The importance of pathogens in tritrophic interactions should not be underestimated, and can have equally important effects as secondary herbivore attackers will have, which are more frequently studied in this context. Plants can often successfully defend themselves against pathogen infection, either in a gene-for-gene interaction or via non-host resistance, resulting, for instance, in the induction of phytohormone signaling pathways, production of reactive oxygen species, and hypersensitive response (Glazebrook, 2005; Nürnberger & Lipka, 2005). Despite this plant-pathogen interaction being less visible to the eye, they lead to major changes in the plant, which can be expected to affect interactions with other community members. Our research demonstrates that it is imperative to work towards greater integration of research on plant-insect and plant-pathogen interactions, as plant defenses to insect and microbial attackers are inexorably linked. This multidisciplinary approach is important to further our knowledge of the modulation of induced plant defenses in response to increasingly complex attack scenarios.

Acknowledgements

We thank Léon Westerd, Frans van Aggelen and André Gidding for culturing the insects, and Wageningen University & Research Centre's experimental farm (Unifarm) for rearing the plants. This work, as part of the European Science Foundation EUROCORES Programme EuroVOL, was supported from funds by the Earth and Life Sciences Council (ALW) of the Netherlands Organisation for Scientific Research (NWO).

Chapter 6

Dual herbivore attack and herbivore density affect metabolic profiles of *Brassica nigra* leaves

Camille Ponzio [1], Stefano Papazian [2], Benedicte Albrectsen [2], Marcel Dicke [1] & Rieta Gols [1]

[1] Laboratory of Entomology, Wageningen University, P.O.Box 16, 6700 AA Wageningen, The Netherlands

[2] Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, 90187 Umeå, Sweden

ABSTRACT

Plant responses to dual herbivore attack are increasingly studied, but effects on the metabolome have largely been restricted to volatile metabolites and defense-related non-volatile metabolites. However, plants subjected to stress, such as herbivory, undergo major changes in both primary and secondary metabolism. Using a naturally occurring system, we investigated metabolome-wide effects of single or dual herbivory on *Brassica nigra* plants by *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars, while also considering the effect of aphid density. Metabolomic analysis of leaf material showed that single and dual herbivory had strong effects on the plant metabolome, with caterpillar feeding having the strongest influence. Additionally, aphid-density-dependent effects were found in both the single and dual infestation scenarios. Multivariate analysis revealed treatment-specific metabolomic profiles, and effects were largely driven by alterations in the glucosinolate and sugar pools. Our work shows that analyzing the plant metabolome as a single entity rather than as individual metabolites provides new insights into the subcellular processes underlying plant defense against multiple herbivore attackers.

Key words: dual herbivory, metabolomics, herbivory density, induced defense, *Pieris brassicae*, *Brevicoryne brassicae*

Introduction

In nature, plants must cope with a large number of attackers, such as insect herbivores and plant pathogens. In response, plants defend themselves via an array of defense strategies, which include both mechanical and chemical defenses, such as the production of secondary plant metabolites (Schoonhoven *et al.*, 2005). Research on plant-insect interactions has largely focused on the effects of a single herbivore attacker, providing a wealth of information on how this interaction will change the plant's phenotype (Kessler & Baldwin, 2002; Mumm & Dicke, 2010; Mithöfer & Boland, 2012), and this at several levels of biological organization (Keurentjes *et al.*, 2011). However, plants are members of complex communities, and can be attacked by a multitude of insect herbivores, often simultaneously or sequentially. Research has increasingly shifted from studying the effects of single to multiple herbivore attack on plant defense mechanisms. Increasing the complexity of the studied interactions can have strong and unpredictable consequences for plant-insect interactions (Dicke *et al.*, 2009), and it is clear that our knowledge of how insect species interact individually with a plant is insufficient to predict the effects of combined herbivore attack at the transcriptional level all the way up to the community level, particularly when the herbivores are of different feeding guilds (De Rijk *et al.*, 2013; Stam *et al.*, 2014).

Plant defenses against insects are regulated by three key signal-transduction pathways each involving a major phytohormone, i.e. jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Chewing herbivores commonly activate the JA-dependent signal-transduction pathway, which often acts synergistically with ET, while the SA pathway is more commonly induced by phloem-feeding insects (Glazebrook, 2005; Pieterse & Dicke, 2007). These signaling pathways are the backbone of a complex regulatory network, and they can interact with each other (Pieterse *et al.*, 2009). Interactions between the SA and JA pathways in particular are often mutually antagonistic, though they can act synergistically or additively as well (Koornneef & Pieterse, 2008; Thaler *et al.*, 2012). While there is a growing pool of studies investigating the effects of multiple herbivory, we still lack in-depth knowledge of the subcellular processes underpinning these more complex interactions. Induction of the defense signaling pathways results in downstream changes in the transcriptome, proteome and metabolome (Kessler & Baldwin, 2002; Keurentjes *et al.*, 2011; Stam *et al.*, 2014), however numerous studies in these fields have mainly taken a highly targeted approach, focusing on specific groups of genes, proteins or metabolites, because of their important defensive functions (Arany *et al.*, 2008; Mithöfer & Boland, 2008; Textor & Gershenzon, 2009; Mathur *et al.*, 2013). However, the plant metabolome

undergoes an extensive rearrangement that will be largely overlooked when using solely a targeted approach. Changes in both primary and secondary metabolism occur in response to insect herbivory, and a whole metabolome analysis can reveal treatment-specific induction patterns (Sutter & Müller, 2011; Kutyniok & Müller, 2012; Ossipov *et al.*, 2014), contributing to a more comprehensive view of plant-herbivore interactions. Moreover, induction of plant responses has been shown to occur locally at the site of damage, as well as systemically, in undamaged leaves (Baldwin *et al.*, 1994; Widarto *et al.*, 2006; Marti *et al.*, 2013).

In the present study, we used a comprehensive metabolomic analysis involving gas-chromatography for general metabolism and liquid-chromatography for mainly glucosinolates (GS) and phenolic compounds to investigate the individual and combined effects of feeding by two insect herbivores of different feeding guilds, i.e. phloem-feeding aphids and leaf-chewing caterpillars, on the plant metabolome. Since these herbivores induce different defense signaling pathways, differential changes in plant chemistry modulate the interaction between the herbivore species, leading to competition or facilitation in the interactions between the two herbivore species (Kaplan & Denno, 2007; Dicke *et al.*, 2009; Soler *et al.*, 2012a). More precisely, the aim was to examine the effects of simultaneous attack by aphids on caterpillar-induced changes in the plant metabolome, and determine if aphid density was an additional contributing factor to any observed changes in the plant's metabolome. This was done on the wild annual black mustard plant *Brassica nigra*, and two of its naturally occurring specialist herbivores: *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars. Previous work on this system has shown that dual attack by *P. brassicae* caterpillars and *B. brassicae* aphids has strong non-linear density-dependent effects on parasitoid foraging behavior (Ponzio *et al.*, 2016). Furthermore, the metabolome of the infested leaves as well as the uninfested adjacent leaves was characterized, in order to determine if changes in the metabolome were present in systemic tissues.

Material and methods

Plants and insects

Brassica nigra plants originated from seeds collected from a local population in Wageningen, the Netherlands, and were grown under greenhouse conditions at 22 ± 2°C, relative humidity (r.h.) of 60-70% and a light:dark regime of L16:D8. *P. brassicae* caterpillars and *B. brassicae* aphids were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv.

Cyrus) in a climate-controlled room (21 ± 1°C, 50-70% r.h., L16:D8).

Experimental treatments

Plants were either undamaged, or subjected to herbivory by either aphids, caterpillars or a combination of both. The following treatments were used: control undamaged plants (C) and plants infested with either 50 aphids (50A), 100 aphids (100A), 30 caterpillars (P), 50 aphids and 30 caterpillars (50A+P), or 100 aphids and 30 caterpillars (100A+P). For the aphid infestations, a combination of first and second instar nymphs were placed on the youngest fully expanded leaf of each plant. After 48h, the three caterpillar treatments were infested with 30 early first-instar caterpillars, on the same leaf as the aphids in the case of the dual-infestation treatments, or on a comparable leaf for the treatment having only caterpillars. All herbivores were left to feed for a further 24h hours before sampling.

The experiments were conducted in a greenhouse under comparable climate conditions as for the plant culture. Six biological replicates were analyzed per treatment, with leaves from nine individual plants (one leaf per plant) pooled per replicate. All treatments were run simultaneously with six runs in total, spread over a period of four weeks. Immediately prior to sampling, all herbivores were removed from the leaves, and their byproducts (frass or honeydew) removed as much as possible with a fine paint brush. For each treatment, two samples were taken from each plant: the 'local', infested leaf, and a younger 'systemic' adjacent leaf. For the control treatment, only one leaf per plant was sampled, at an intermediary position. Leaves were detached using a surgical blade and the nine leaves constituting one biological replicate were wrapped together in aluminum foil before being flash frozen in liquid nitrogen and subsequently stored at -80°C. Samples were then ground with a mortar and pestle in liquid nitrogen, and were shipped to Umeå University, Sweden, on dry ice for analysis.

Metabolomics

Extraction

Freeze-dried leaf samples of 10-12 mg were extracted using 1 ml of cold chloroform:methanol:H_2O (20:60:20), which contained 7.5 ng/µl salicylic acid as an internal standard for both GC and LC -MS. Other internal standards included in the extraction buffer for quality control (GC-MS detection) were salicylic acid-D6, methyl stearate-^{13}C4, hexadecanoic acid ^{13}C4, succinic acid-D4, α-ketoglutarate-^{13}C4, L-glutamic acid-^{13}C5-^{15}N, myristic acid-^{13}C, and putrescine-D4. A 3 mm tungsten carbide bead was

introduced in each vial, and samples were agitated for 3 min at 30 Hz in an MM 301 Vibration Mill (Retsch GmbH and Co. KG, Haan, Germany). Extracts were centrifuged at 20,800 × *g* for 10 min at 4°C in order to remove tissue debris from the mixture and to avoid contamination. 200µl of the supernatant were evaporated until dry using a SpeedVac™. For LC-MS analysis, dried samples were re-dissolved in 10 µl of cold methanol and diluted with 10 µl of cold water prior to being injected into the system. For GC-MS analysis, samples were further derivatized using 30 µl of methoxyamine (15 µg/µL in pyridine), agitated for 10 min, allowed to react for 16 hours at 25°C. Sylilation was done with 30 µl of MSTFA and reacted for 1 hour at 25°C. Lastly, GC-MS samples were diluted in 30 µl of heptane which contained 15 ng/µl of methyl stearate (internal standard), and then injected into the system.

Gas Chromatography - Mass Spectrometry (GC-MS)

One microliter of the derivatized sample was injected splitless (or split 1:20) by a CTC Combi Pal Xt Duo (CTC Analytics AG, Switzerland) auto-sampler/robot into an Agilent 7890A gas chromatograph equipped with a 30 m × 0.25 mm i.d. fused-silica capillary column with a chemically bonded 0.25-µm DB 5-MS UI stationary phase (J&W Scientific, Folsom, CA). The injector temperature was 260 °C, the purge flow rate was 20 ml/min, and the purge was activated after 75 s. The gas flow rate through the column was 1 ml/min, and the column temperature was held at 70 °C for 2 min, followed by an increase of 20 °C/min to 320 °C, and this temperature was held for 8 minutes. The column effluent was added into the ion source of a Pegasus HT time-of-flight mass spectrometer, GC/TOF-MS (Leco Corp., St Joseph, MI). The transfer line was at a temperature of 250 °C, and the ion source temperature was at 200 °C. Ions were generated by a 70-eV electron beam at an ionization current of 2.0 mA, and 20 spectra/s were recorded in the mass range 50−800 m/z. The acceleration voltage was turned on after a solvent delay of 290 s. The detector voltage was 1500-2000V.

Liquid Chromatography - Mass Spectrometry (LC-MS)

Chromatographic separation was done on an Agilent 1290 Infinity UHPLC-system (Agilent Technologies, Waldbronn, Germany). 2 µl of extracted leaf samples (re-suspended aliquots) were injected onto an Acquity UPLC HSS T3, 2.1 x 50 mm, 1.8 µm C18 column combined with a 2.1 mm x 5 mm, 1.8 µm VanGuard precolumn (Waters Corporation, Milford, MA, USA) held at 40 °C. The gradient elution buffers used were A (H_2O, 0.1 % formic acid) and B (75/25 acetonitrile:2-propanol, 0.1 % formic acid), and the flow-rate was 0.5 ml/min. The compounds were eluted with a linear gradient consisting of 0.1 -

10 % B over 2 minutes, B was increased to 99 % over 5 minutes and held at 99 % for 2 minutes; B was decreased to 0.1 % for 0.3 minutes and the flow-rate was increased to 0.8 ml/min for 0.5 minutes; these conditions were held for 0.9 minutes, after which the flow rate was reduced to 0.5 ml/min for 0.1 min before the next injection.

The compounds were detected with an Agilent 6540 Q-TOF mass spectrometer equipped with a jet stream electrospray ion source operating in negative ionization mode. A reference interface was connected for accurate mass measurements; the reference ions purine (4 μM) and HP-0921 (Hexakis(^{1}H, ^{1}H, ^{3}H-tetrafluoropropoxy)phosphazine) (1 μM) both purchased from Agilent Technologies (Santa Clara, CA, USA) were infused directly into the MS at a flow rate of 0.05 ml/min for internal calibration, and the monitored ions were purine (m/z 119.03632); HP-0921 (m/z 966.000725). The gas temperature was set to 300 °C, the drying gas flow to 8 l/min and the nebulizer pressure 40 psig. The sheath gas temp was set to 350°C and the sheath gas flow 11 l/min. The capillary voltage was set to 4000 V in positive ion mode, and to 4000 V in negative ion mode. The nozzle voltage was 0 V. The fragmentor voltage was 100 V, the skimmer 45 V and the OCT 1 RF Vpp 750 V. The collision energy was set to 0 V while the m/z range was 70 – 1700. Data were collected in centroid mode with an acquisition rate of 4 scans/s.

Orbitrap Tandem Mass Spectrometry (MS-MS)

In order to verify the data acquired by the UHPLC-ESI/TOF-MS, samples were re-analyzed by UHPLC-MS-MS using linear ion trap (LTQ-Orbitrap). Separation was performed on a Thermo Accela LC system, equipped with a column oven (held at 40°C) and a Hypersil C18 GOLD™ column (2.1×50 mm, 1.9 μm; mobile phase as for the UHPLC-ESI/TOFMS) and analyzed by tandem mass spectrometry using a LTQ/Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). External mass calibration was performed according to the manufacturer's guidelines.

Compounds identification and data processing

For GC-MS, identification of compounds was based on comparison of retention indices (RIs) and mass spectra libraries with the Swedish Metabolomics Centre in-house database and the public Golm Metabolome Database of the Max Planck Institute. RIs were calculated relatively to the C8-C40 alkane series which was included in the analysis. Feature extraction and peak integration from the raw data were performed in Matlab® environment. Sample were normalized by PCA UVN scores of the values integrated for the internal standards (methyl stearate, salicylic acid-D6, methyl stearate-^{13}C4, hexadecanoic

acid ^{13}C4, succinic acid-D4, α-ketoglutarate-^{13}C4, L-glutamic acid-^{13}C5-15N, myristic acid-^{13}C, and putrescine-D4).

For LC-MS, Mass Feature Extraction (MFE) for the data acquired was performed using the MassHunter™ Qualitative Analysis software package, version B06.00 (Agilent Technologies Inc., Santa Clara, CA, USA). Extracted features were aligned and matched between samples using Mass Profiler Professional™ 12.5 (Agilent Technologies Inc., Santa Clara, CA, USA). Compound identities were then compared to pure glucosinolate standards (Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) (sinigrin, glucoiberin, glucobrassicin, gluconapin, glucobrassicanapin, glucotropaeolin, gluconasturtin, progoitrin), METLIN mass spectra depository, and additional literature references for glucosinolates (Clarke, 2010), and for flavonol glucosides and hydroxycinnamic acid derivatives (Lin *et al.*, 2011). Additional tandem mass data analysis by Orbitrap was used to compare the MS^n profiles and confirm the list of identifications. Raw data were processed using Sieve® and Matlab® software for peak alignment and integration. Normalization was performed for the integrated area of the labeled internal standards (salicylic acid-D6, m/z [M-H-] 141.046).

Statistical analysis

The metabolic profiles were analysed through multivariate data analysis using OPLS-DA (orthogonal projection to latent structures discriminant analysis) (Eriksson *et al.*, 2006), executed with the SIMCA-14 software program (Umetrics AB, Umeå, Sweden). This projection method determines whether samples can be separated based on differences in the overall metabolite profiles. OPLS-DA computes the part of variation of X (matrix with metabolites) which is predictive (correlated to Y, with Y being a data matrix of dummy variables assigning a sample to it respective class, here treatment) and the variation which is orthogonal (unrelated) to Y (Trygg & Wold, 2002), as removal of non-correlated variation from the PLS model enhances its interpretability. The results of the analysis are then visualized in score plots, which show the sample structure according to latent variables, and loading plots, which display the contribution of the variables to these latent variables as well as the relationships among the variables. For the pairwise comparisons presented in Table 1, the number of orthogonal latent variables was standardized to a maximum of three latent variables, in order to facilitate comparisons across the different pairwise models and to minimize the risk of overfitting the models. In order to identify the most highly influential metabolites in the different models, the variable importance in the projection (VIP) of each variable (in this case, the different compounds) was used, with variables having VIP values greater than 1 being most influential in the model

(Eriksson *et al.*, 2006). Data were mean-centred and scaled to unit variance before they were subjected to the analysis. All unknown metabolites were included from the analysis, but subsequently masked in the loading plots in order to increase the interpretability of the plots. Individual metabolites and total glucosinolates were analysed in SPPS 22 (IBM, Armonk, NY, USA) by one-way ANOVA followed by Tukey's posthoc test. Following inspection of the residuals, data that did not follow a normal distribution were log or square root transformed. If only the assumption of equal variances was violated, then a one way Welch-ANOVA was done, followed by Games-Howell post-hoc test, which is suited to data with unequal variances.

Results

The metabolomic analysis resulted in the detection of 221 compounds, 110 of which could be fully identified, covering both primary and secondary metabolism. The major classes of identified compounds were amino acids, sugars, acids, glucosinolates (GS), hydroxycinnamic acid derivatives and flavonol glucosides. (Tables S1 and S2) In general, insect herbivory altered the plants' metabolome, with the strongest differences seen between control and treated plants (Fig. 1), and smaller differences between the infestation treatments. These general and treatment-specific effects were much stronger in the local leaves of insect-damaged plants (Fig. 1) than for the systemic leaf samples (not shown), which resulted in a weak and unreliable model where control plants could not be adequately separated from the 'systemic' samples from treated plants.

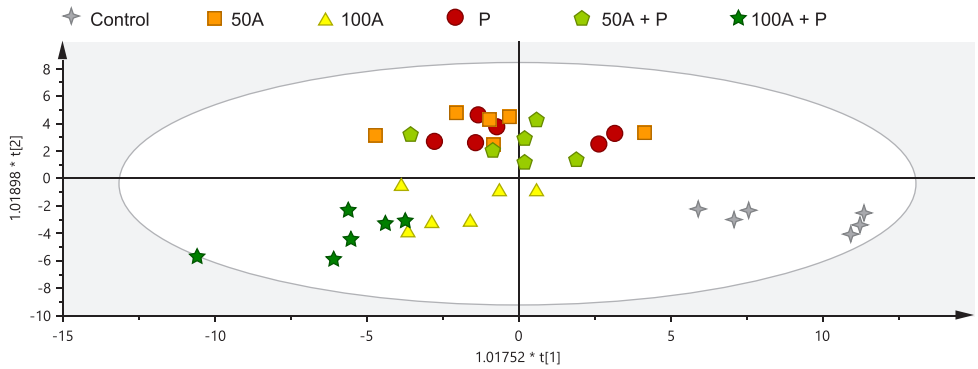


Figure 1. Multivariate analysis (OPLS-DA, score plot) of *B. nigra* leaf metabolic profiles, describing the effect of aphid and caterpillar herbivory in the local, treated leaf. OPLS-DA: 3+2+0 latent variables, with $R^2X = 0.572$ $R^2Y = 0.519$ $Q^2 = 0.213$. Symbols in the score plot refer to healthy plants (C), plants infested with 50 *B. brassicae* aphids (50A), 100 aphids (100A), *P. brassicae* caterpillars (P), 50 aphids plus caterpillars (50A+P) or 100 aphids plus caterpillars (100A+P). The ellipse defines Hotelling's T2 confidence region (95 %).

When the metabolomes of herbivore-infested plants were compared to each other in a pairwise fashion for the local and systemic samples separately (Table 1), 5 out of 6 OPLS-DA comparisons resulted in statistically significant separation of the treatments for the local tissue samples, while for the systemic samples this was only the case for 2 out of 6 comparisons. Examination of the loading plots (Fig. S1) and associated VIP value lists of the 'local' models showed that it was rarely the same individual compound or set of compounds that contributed to the pairwise differences across the different comparisons. For instance, when comparing the 50 and 100 aphids density conditions, with and without caterpillars – i.e. 50A versus 50AP (model 5) and 100A versus 100AP (model 6) - the group of discriminant metabolites (VIP list) in each case was highly divergent: indeed, while the effect of *Pieris* caterpillars on plants treated with low-density infestation of aphids (50 aphids) mainly resulted in changes in sugars and phenolic compounds (flavonol glucosides and hydroxycinnamic acid derivatives), the effect of *Pieris* feeding on plants with a high-density aphid infestation (100 aphids) was explained by GS (mainly glucobrassicin and glucoiberin) and many unknown compounds (Fig. S1). On the other hand, comparison of the two systemic treatment pairs showed a strong overlap in the highly discriminant metabolites, particularly reduced glutathione (GSH), phenylalanine, three flavonol glucosides and several unknown compounds (Fig.S1).

Table 1. Overview of pairwise comparisons using multivariate data analysis (OPLS-DA) in the local and systemic leaf samples, with orthogonal variation standardized at maximum 3 latent variables. Model parameters were included for all comparisons where a separation between treatments was possible.

			Model parameters			
Model number	Treatment A	Treatment B	Latent variables	$R^2X(cum)$	$R^2Y(cum)$	$Q^2(cum)$
Local leaf samples						
1	50A	100A	1+3+0	0.625	0.998	0.515
2	50A+P	100A+P	1+0+0	0.306	0.576	0.192
3	P	50A+P	-	-	-	-
4	P	100A+P	1+3+0	0.676	0.991	0.137
5	50A	50A+P	1+3+0	0.524	0.984	0.365
6	100A	100A+P	1+2+0	0.521	0.985	0.391
Systemic leaf samples						
7	50A	100A	-	-	-	-
8	50A+P	100A+P	1+3+0	0.645	0.997	0.378
9	P	50A+P	1+1+0	0.687	0.998	0.560
10	P	100A+P	-	-	-	-
11	50A	50A+P	-	-	-	-
12	100A	100A+P	-	-	-	-

The difference between these local and systemic effects (Fig. 1 and Table 1) indicated that herbivory, either - single or dual - indeed induced metabolomic changes in adjacent undamaged leaves, but these effects were weaker compared to the effects on the local leaf (i.e. directly damaged tissues). These results motivated the decision to perform further statistical analyses which focused exclusively on the local leaf samples, and which are the focus of the remainder of this section.

More subtle effects of the aphid treatments on local leaves were detected by excluding aphid density from the explanatory variables. In this way differences could be detected based on the binary absence or presence of aphids or caterpillars. In this model, the projection showed a separation on the first latent variable (LV) as an effect of caterpillar herbivory, separating plants infested only with aphids from caterpillar-infested plants (Fig. 2a). Plants that were simultaneously damaged by both herbivores could not be separated from plants which were infested with only caterpillars, indicating that caterpillar herbivory had a stronger effect on the plant metabolome than aphid infestation (Fig. 2a). The corresponding loading plot (Fig. 2b) and VIP plots showed that aphid-only-infested plants generally contained more sugars, particularly fructose, trehalose maltose/ cellobiose, ribitol, glucose and sorbose, as well as GSH, α-tocopherol, p-coumaric-acid, and Qn-3-coumaroylsophoroside-7-glucoside. The caterpillar-infested treatments were primarily characterized by higher levels of many GS, namely glucoiberin, gluconapin, glucobrassicin, neoglucobrassicin, sinigrin and gluconasturtin. However, after dividing the dataset into the two aphid density subsets (50 and 100 aphids) and modeling the effects separately for each aphid density, the overall picture of aphid-induced effects could be further refined (Fig. 3a). At the higher aphid density, a strong separation was visible between the 100 aphids-, caterpillar- and dual infestation treatments, with the first LV corresponded to the effect of the caterpillars on plant metabolome. Metabolites which contributed strongly to the separation were eight GS (with only 4-methoxy-glucobrassicin primarily associated to the dual infestation treatment), glycolic acid, phosphoric acid, GSH and two sugars (fructose and trehalose). However, a significant overlap among the treatments was found when the same analysis was performed on the lower aphid density infestation (50 aphids), and hence no model could be created, indicating that the effects of aphid infestation on caterpillar induction was density dependent. Thus, aphid infestation had an effect on the plant metabolomic response to herbivory by caterpillars, and this effect was dependent on aphid density, but the effect of caterpillar herbivory was always stronger compared to the overall effect of aphids, which was only clearly detected at the higher aphid density.

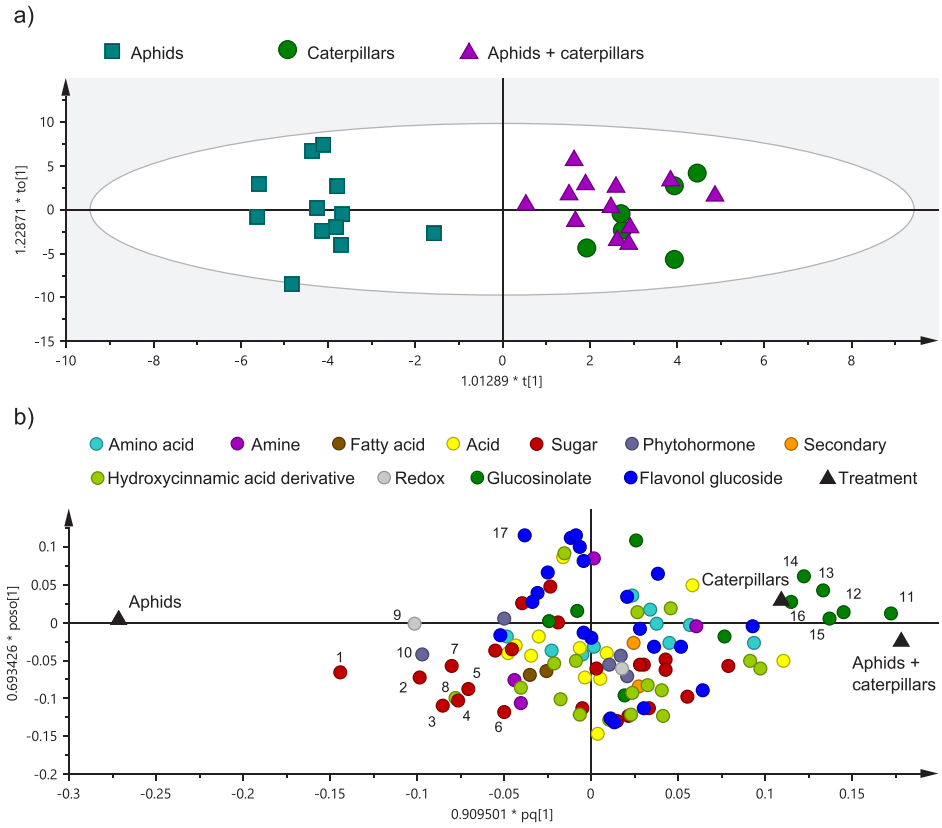


Figure 2. Multivariate analysis (OPLS-DA, scores (a) and loadings (b)) of *B. nigra* leaf metabolic profiles, describing the effect of aphid and caterpillar herbivory in the local, infested leaf. OPLS-DA: 1+1+0 latent variables, with $R^2X = 0.121$ $R^2Y = 0.494$ $Q^2 = 0.201$. Symbols in the score plot (a) refer to plants infested with *B. brassicae* aphids, *P. brassicae* caterpillars or aphids plus caterpillars. The ellipse defines Hotelling's T2 confidence region (95 %), and a second latent variable was added for representational purposes. (b) Loading plot of the first two components of the OPLS-DA, showing the contribution of each of the individual metabolites towards the model. Numbers refer to the highly discriminatory metabolites: (1) fructose, (2) fructose/sorbose, (3) maltose/cellobiose, (4) ribitol, (5) glucose, (6) trehalose, (7) sorbose, (8) p-coumaric-acid, (9) GSH, (10) α-tocopherol, (11) glucoiberin, (12) gluconapin, (13) glucobrassicin, (14) neoglucobrassicin, (15) sinigrin, (16) gluconasturtin and (17) Qn-3-coumaroylsophoroside-7-glucoside.

Minor effects were better visualized when the number of treatments compared was decreased. As the caterpillar herbivory treatment (P) had a strong influence on the models, it was then omitted from the analyses in order to reveal potential minor density-dependent effects of aphid infestation in the single and dual stress treatments (Fig. 4). In this model, the projection showed an effect of both caterpillar herbivory and aphid density (Fig. 4a). A first LV separated the treatments according to aphid density and a second LV separated plants subjected to caterpillar herbivory from plants without

caterpillar herbivory. This approach confirmed the density effects already observed in the previous comparison between plants with 100 aphids, caterpillars or both (Fig.3). One of the main discriminatory groups of metabolites in this model was the GS (Fig. 4b). Seven of these were predominant in both caterpillar-infested treatments (in order of importance; glucoiberin, sinigrin, gluconapin, glucobrassicin, neoglucobrassicin, 4-hydroxy-glucobrassicin and gluconasturtin). On the other hand, single infestation by aphids generally led to higher levels of sugars. A density-dependent effect was observed on some of these sugars, with the 100-aphid treatment leading to a higher content of ribose,

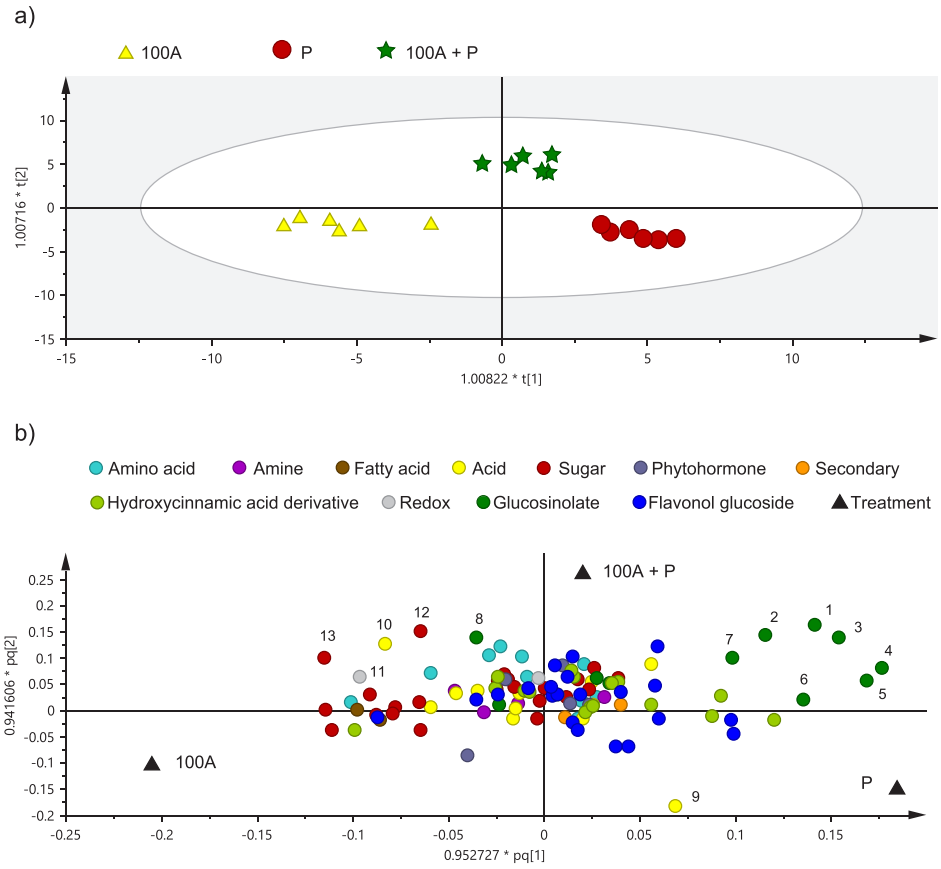


Figure 3. Multivariate analysis (OPLS-DA, scores (a) and loadings (b)) of *B. nigra* leaf metabolic profiles, describing the effect of aphid and caterpillar herbivory in the local, infested leaf, at the higher aphid density. OPLS-DA: 2+3+0 latent variables, with $R^2X = 0.643$ $R^2Y = 0.951$ $Q^2 = 0.4$. Symbols in the score plot (a) refer to plants infested with 100 *B. brassicae* aphids (100A), *P. brassicae* caterpillars (P), or 100 aphids plus caterpillars (100A+P). The ellipse defines Hotelling's T2 confidence region (95 %), and a second latent variable was added for representational purposes. (b) Loading plot of the first two components of the OPLS-DA, showing the contribution of each of the individual metabolites towards the model. Numbers refer to the highly discriminatory metabolites: (1) glucoiberin, (2) neoglucobrassicin, (3) glucobrassicin, (4) sinigrin, (5) gluconapin, (6) gluconasturtin, (7) 4-hydroxy-glucobrassicin, (8) 4-methoxy-glucobrassicin, (9) glycolic acid, (10) phosphoric acid, (11) GSH, (12) fructose, and (13) trehalose.

ribitol, trehalose, maltose and xylose, while the 50-aphid treatment was characterized by increased levels of fructose. Plants that were single or dual infested with 100 aphids also showed an increasing trend of the two fatty-acid compounds, namely cis,cis-linoleic acid (octadecadienoic acid, 9,12-[*Z,Z*]-) and α-linolenic acid (octadecatrienoic acid, 9,12,15-[*Z,Z,Z*]-), while plants with the lower aphid density showed increased levels of glycolic acid in both single and dual infestation treatments.

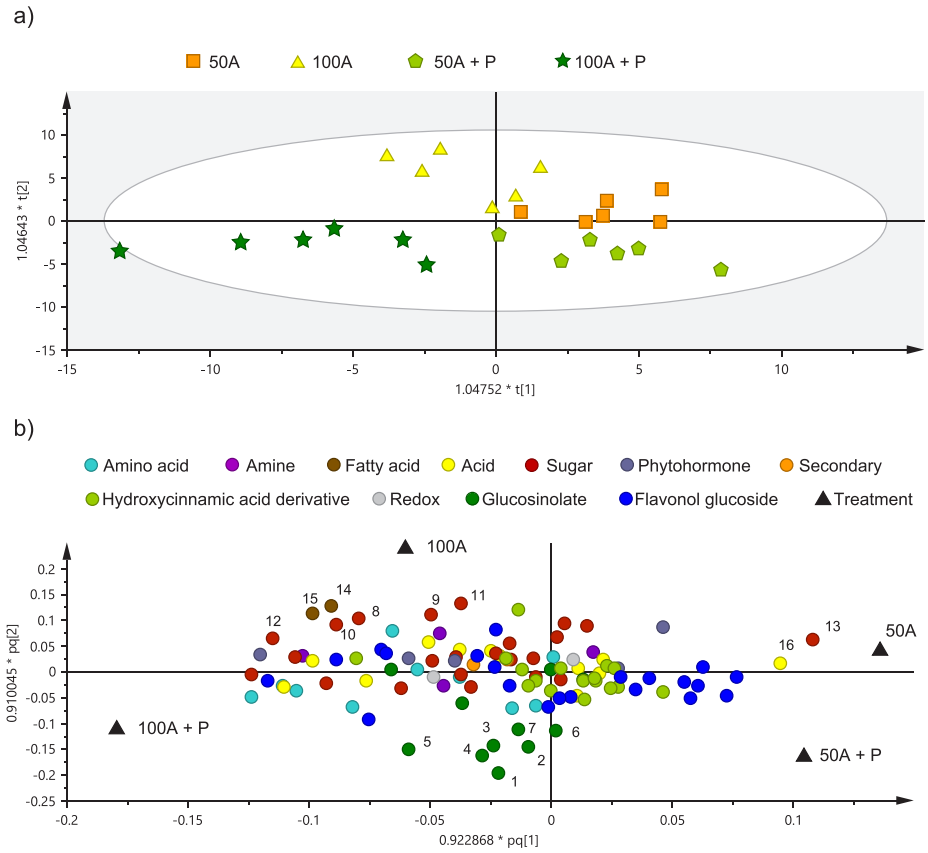


Figure 4. Multivariate analysis (OPLS-DA, scores (a) and loadings (b)) of *B. nigra* leaf metabolic profiles, describing the effect of aphid density. OPLS-DA: 1+4+0 latent variables, with $R^2X = 0.525$ $R^2Y = 0.746$ $Q^2 = 0.155$. Symbols in the score plot (a) refer to plants infested with 50 *B. brassicae* aphids (50A), 100 aphids (100A), 50 aphids plus *P. brassicae* caterpillars (50A+P) or 100 aphids plus caterpillars (100A+P). The ellipse defines Hotelling's T2 confidence region (95 %), and a second latent variable was added for representational purposes. (b) Loading plot of the first two components of the OPLS-DA, showing the contribution of each of the individual metabolites towards the model. Numbers refer to the highly discriminatory metabolites: (1) glucoiberin, (2) sinigrin, (3) gluconapin, (4) glucobrassicin, (5) neoglucobrassicin, (6) 4-hydroxy-glucobrassicin, (7) gluconasturtin, (8) ribose, (9) ribitol, (10) trehalose, (11) maltose/cellobiose (12) xylose, (13) fructose, (14) cis,cis-linoleic acid, (15) α-linolenic acid, and (16) glycolic acid.

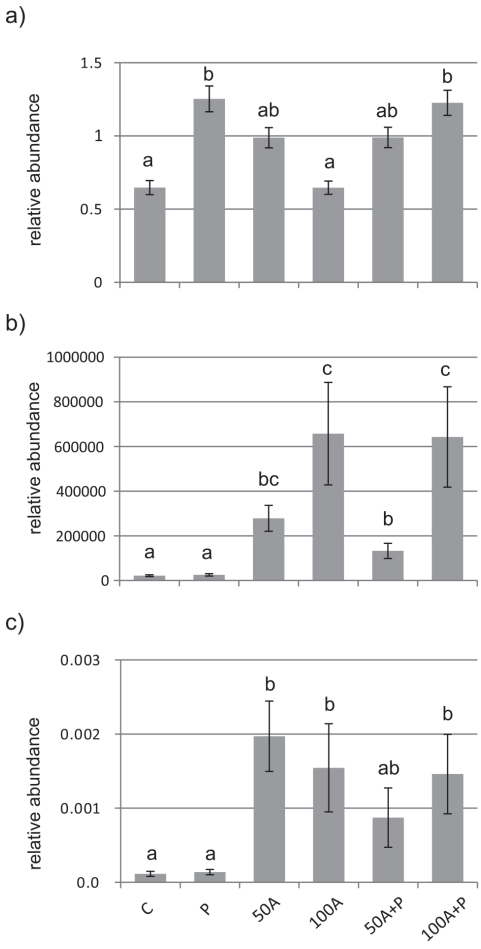


Figure 5. Relative abundance (mean ± SE, N=6) of (a) total glucosinolates, (b) trehalose and (c) glutathione GSH in *B. nigra* plants under the different herbivory treatments. Different letters over the bars indicate significant differences.

The effects of compounds which appeared to have strong discriminatory effects in several models were further investigated with univariate analysis (Fig. 5). Although OPLS-DA loading plots (Figs. 2b and 4b) showed few GS to be affected in a highly treatment-specific manner, the whole GS pool tended to be affected as a group. Analysis by ANOVA indicated that aliphatic and indolic GS were affected in the same way when these two GS groups were analyzed separately, so only the effects on total GS are reported here (Fig. 5a). While herbivory generally led to an increase in the GS pool, aphid density did not affect GS levels significantly in this study (Welch ANOVA, df = 5, F = 11.382, $P < 0.001$). There was an interactive effect of caterpillar herbivory and aphid density in the dual treatments: while infestation with 100 aphids did not affect caterpillar-induced changes in GS content, co-infestation with 50 aphids led to reduced GS concentrations compared to caterpillar herbivory alone. The sugar trehalose (Fig. 5b) was another important compound in separating the different treatments. Levels of this compound did not increase after caterpillar herbivory alone, but showed a strong increase in both the single and dual aphid-infestation treatments (One-way ANOVA, df = 5, F = 18.23, $P < 0.001$). Furthermore, these effects were aphid-density dependent, with the highest levels of trehalose found following infestation with 100 aphids. Glutathione GSH (Fig. 5c) was also found to be strongly induced by aphid infestation (One-way ANOVA, df = 5, F = 7.59 $P < 0.001$), whether alone or in co-infestation with caterpillars, but not induced by caterpillar herbivory alone.

Discussion

Our data show that the metabolome of *B. nigra* plants was affected by feeding by aphids or caterpillars, both in single infestations and when both species were present simultaneously. The effects of caterpillar herbivory were the strongest effects and largely overrode aphid-induced changes. Despite this strong influence of caterpillar herbivory, aphid density-dependent effects on caterpillar induction were detected as well. Co-infestation by the aphid and caterpillar species used in this study have previously been shown to affect the foraging behavior of parasitoid wasps (Ponzio *et al.*, 2016). These results motivated the largely untargeted metabolome approach of this study, which was performed in order to gain a comprehensive overview of the chemical changes that are involved in *B. nigra* responses to dual herbivory, coupled to density effects. Although literature on this topic is limited, both caterpillar and aphid herbivory are known to induce changes in the plant metabolome, with aphid-induced effects of a lower magnitude. Caterpillar herbivory often leads to large scale changes in metabolic profiles (Kersten *et al.*, 2013), though these effects can vary according to the species (Steinbrenner *et al.*, 2011), specialization (generalist versus specialist) (Sutter & Müller, 2011), or larval instar (Widarto *et al.*, 2006). In contrast, aphid infestation triggers metabolome changes on a smaller scale (Sutter & Müller, 2011; Kutyniok & Müller, 2012), which may be a result of the minimal damage caused by the piercing/sucking feeding mode. This division between leaf chewers and sap-sucking herbivores is also typical in the induced volatile metabolome (Rowen & Kaplan, 2016), indicating that feeding guild has a strong impact on induced plant defenses. The difference in strength of effect by the two herbivore species in our study may account for the overriding effects of caterpillar herbivory we found.

While there is some information on the impact of single herbivorous attackers on the metabolome, studies are usually focused on investigating specific metabolite pools, and the effects of dual attack or herbivore density is still largely uncharted territory. Kutyniok & Müller (2012) showed that while aphid infestation did change the plant's metabolic fingerprint, combined attack with root-feeding nematodes resulted in no measurable effects. In another recent study, Khaling *et al.* (2015) examined the effects of combined *P. brassicae* caterpillar herbivory and ozone exposure on pools of defense-related metabolites. While the three main compound groups showed signifcant effects of the single stresses, few interactive effects of ozone and herbivore were detected. However, interaction of the stresses was detected on some individual metabolites, namely two GS which were reduced by exposure to dual stress, and two unknown peaks induced by ozone that were affected in opposite directions by dual stress with caterpillars. Although in the present

study the effect of aphid infestation was minor compared to caterpillar infestation, a density-dependent effect was detected between plants infested with 50 or 100 aphids, either as a single stressor or in combination with caterpillar herbivory. Aphids generally had a stronger effect on the overall metabolite profile at the higher density only, which affected induction by caterpillar herbivory. Herbivore-density-dependent effects on plant metabolome were previously shown for geometrid caterpillars (*Epirrita autumna*) feeding on mountain birch trees (*Betula pubescens* ssp. *czerepanovii*) (Ossipov *et al.*, 2014): increasing density was linked to important biochemical changes, including an increase in secondary compounds (phenolics), and decrease of primary metabolism (nutritive metabolites).

Effects on plant secondary metabolism

One of the most strongly affected metabolite pools was the GS, which are an important group of secondary defense compounds characteristic of the Brassicaceae that mediate interactions with other organisms including insects (Chew, 1988; Gols & Harvey, 2009; Hopkins *et al.*, 2009). After tissue damage, these compounds are hydrolyzed by myrosinase enzymes into toxic nitrogen- or sulfur-containing breakdown products (Halkier & Gershenzon, 2006). GS hydrolysis products are an important defense against many generalist herbivores, while specialist herbivores, like those studied here, have evolved adaptations to detoxify, sequester or excrete GS (Rask *et al.*, 2000; Ratzka *et al.*, 2002). Insect herbivory on brassicaceous plants will most frequently induce indolic GS, while aliphatic and aromatic GS are induced at lower magnitudes, and concentrations may even drop following herbivory (reviewed by Textor & Gershenzon, 2009). Aliphatic GS appear more affected by aphid herbivory than indolic GS, with aphid feeding often leading to reduced induction of aliphatic GS (Kim & Jander, 2007; Kusnierczyk *et al.*, 2008; Kutyniok & Müller, 2012). However, we found that levels of both aliphatic and indolic GS followed the same treatment-specific pattern of induction, and GS were little induced by aphid feeding at the higher density. Interestingly, during dual attack with caterpillars there was an interactive effect of caterpillar feeding and aphid density: dual infestation with 100 aphids did not affect caterpillar-induced GS levels, but the lower 50 aphid density did. Studies investigating GS concentrations during dual attack found that there was no effect of dual caterpillar herbivory by *P. brassicae* and *B. brassicae* on cultivated *Brassica oleracea* GS content (Soler *et al.*, 2012a), and no effect of dual stress with *P. brassicae* and ozone exposure in *B. nigra* (Khaling *et al.*, 2015). Combined attack by flea beetles (*Phyllotreta nemorum*) and an oomycete pathogen (*Albugo sp.*) had an additive effect on GS induction in the brassicaceous plant *Barbarea vulgaris* (van Mölken *et al.*, 2014). However, in all the above studies, attacker density effects have not been considered, whereas our study

has shown that in order to determine the potential effects of dual attack, herbivore density must be considered as is may influence the strength and direction of plant responses.

Effects on plant primary metabolism

Along with the GS, sugars were another class of metabolites which were strongly affected by herbivory. Feeding by *B. brassicae* induced a strong accumulation of many sugars in *B. nigra* plants, and fructose and trehalose were positively correlated to aphid density. This was shown previously for trehalose in *Myzus persicae*-infested *Arabidopsis* plants (Hodge *et al.*, 2013), in both the infested and uninfested leaves, though it can also be present only in the infested leaves (Singh *et al.*, 2011). In our case, trehalose was present only in the aphid-infested samples and was absent from the systemic samples, it cannot be excluded that this metabolite was present in the aphid honeydew rather than in the plant, though it may be that aphids concentrate and excrete plant-derived trehalose. This may also be the case for other sugars that were significantly affected by aphid herbivory (fructose, sucrose), as in *Tuberculatus quercicola* aphids, trehalose, glucose, fructose, sucrose and melezitose together represented around 90% of the honeydew's total sugar content (Yao & Akimoto, 2001). Trehalose is called a 'protective sugar', since it is involved in induced plant responses against (a)biotic stresses, conferring tolerance to drought (Fernandez *et al.*, 2010), and accumulating in plant tissues in response to pathogen infection or infestation by phloem-feeding insects (Fernandez *et al.*, 2010; Singh & Shah, 2012). Plants under (a)biotic stress produce reactive oxygen species, and trehalose protects against oxidative damage (Fernandez *et al.*, 2010). Interestingly, glutathione GSH followed a similar pattern of induction; it is strongly induced by aphid herbivory but not by caterpillar herbivory. Glutathione (both reduced glutathione (GSH) and oxidized glutathione (GSSG)) is referred to as the "master antioxidant", protecting cells from oxidative damage during biotic or abiotic stress. It is known to be involved in defense responses against phytopathogens via cross-communication with the salicylic-acid signaling pathway (Mou *et al.*, 2003; Ghanta *et al.*, 2011), though it can also be active against insect herbivory, though in resistant plants (Liu *et al.*, 2015). While only a snapshot of the many primary metabolites affected by herbivory and herbivore density, these results provide important insights into plant responses to herbivory, as they show that it is important to consider all components of the metabolome, and not only the commonly studied secondary defense-related metabolites. Elements of the primary metabolism may serve key roles in plant defense, and plant tolerance/resistance to herbivory requires changes in both primary and secondary metabolism.

Conclusions

Our results show that herbivory by *P. brassicae* caterpillars and *B. brassicae* aphids was associated with multiple changes in the leaf metabolome. While caterpillar herbivory had the strongest effect, there was an interactive effect of infestation by the two herbivores, and this effect was aphid-density-dependent. While this study focused on the identified metabolites, numerous other unidentified metabolites were also strongly affected by dual herbivory, some also in a density-dependent manner, and they may play important roles in defense against herbivores, and so these may warrant further investigation. Untargeted metabolomics is a powerful tool that deserves to be further exploited in interactions of plants with multiple herbivores. Consideration of a single compound class provides important insight in plant modulation of defense responses, but fails to provide a clear picture of the global metabolic plant response to one or several attackers. Herbivory does not lead exclusively to changes in secondary plant metabolism; primary metabolism undergoes important changes as well, showing effects on the allocation of resources, and highlighting primary metabolites which may even have defensive functions themselves or modulate defense signaling pathways (Schwachtje & Baldwin, 2008; Steinbrenner *et al.*, 2011). Such an untargeted or wide approach warrants further attention as organisms interacting with plants usually are affected by mixtures of metabolite changes, and not only variation in concentrations of individual compounds. In this sense, many minor changes in a multitude of compounds which may not be significantly affected individually, as seen here, may have stronger effects on interacting organisms than limited changes in only a few important targeted compounds, as found for the volatile metabolome of *B. nigra* (Ponzio *et al.*, 2014; Ponzio *et al.*, 2016). We showed that multivariate analysis is a valuable tool for gaining a global perspective on complex plant-insect interaction scenarios, such as dual herbivory, at the '-omics' level.

Acknowledgements

We thank Léon Westerd, Frans van Aggelen and André Gidding for culturing the insects, and Wageningen University & Research Centre's experimental farm (Unifarm) for rearing the plants. We also thank the Swedish Metabolomic Centre in Umeå, Sweden for support in performing the metabolic analyses. This work, as part of the European Science Foundation EUROCORES Programme EuroVOL Consortium A-BIO-VOC was supported by funds from the Earth and Life Sciences Council (ALW) of the Netherlands Organisation for Scientific Research (NWO) and from the Swedish Research Council (VR).

Supporting information

Table S1. Identified and unknown metabolites from GC-TOF-MS analysis. Compound class, retention index (RI) and compound identity are reported.

Compound class	RI	Compound ID
Amino Acid	1105.9	Alanine
Amino Acid	1421.5	β-alanine
Amino Acid	1658.4	Asparagine
Amino Acid	1302.1	Glycine
Amino Acid	1762.8	Glutamine
Amino Acid	1287.4	Isoleucine
Amino Acid	1623.4	Phenylalanine
Amino Acid	1350.3	Serine
Amino Acid	1375.2	Threonine
Amino Acid	1212.9	Valine
Amine	1239.8	Urea
Amine	2094.8	N-acetyl mannosamine
Amine	2124.3	N-acetyl mannosamine
Amine	2249.9	Spermidine
Fatty acid	2207.2	Octadecadienoic acid, 9,12-(*Z,Z*)-
Fatty acid	2214.2	Octadecatrienoic acid, 9,12,15-(*Z,Z,Z*)-
Acids	1803.6	Citric acid
Acids	1342.8	Fumaric acid
Acids	1477.4	Malic acid
Acids	1319.6	Glyceric acid
Acids	1078.9	Glycolic acid
Acids	1132.9	Oxalic acid
Acids	1543.4	Threonic acid
Acids	1544.5	Threonic acid
Acids	1369.3	Threonic acid lactone
Acids	1982.5	Galactonic acid
Acids	1912.3	Glucuronic acid-e-lactone
Acids	1264.9	Phosphoric acid
Sugars	2728.1	Trehalose
Sugars	1882.2	Glucose
Sugars	2619	Sucrose
Sugars	2621.2	Sucrose
Sugars	1856.8	Fructose
Sugars	1866.7	Fructose
Sugars	1863.8	Fructose or Sorbose
Sugars	1953.8	Glucopyranose
Sugars	1979.5	Glucopyranose

Compound class	RI	Compound ID
Sugars	1464.3	Erythrose
Sugars	1869.2	Mannose
Sugars	3368.4	Raffinose
Sugars	1682.7	Ribose
Sugars	1854.9	Sorbose
Sugars	1637.7	Xylose
Sugars	1643.4	Xylose
Sugars	1659.9	Xylose
Sugars	2721.5	Maltose or Cellobiose
Sugars	2977.2	Galactinol
Sugars	2080.9	Myo-inositol
Sugars	1738.3	Ribitol
Sugars	1929.4	Sorbitol
Sugars	1708.3	Xylitol
Phytohormone	3143.1	α-tocopherol
Phytohormone	3277.3	Campesterol
Phytohormone	3359.8	β-sitosterol
Phytohormone	3361.4	β-sitosterol
Phytohormone	2166.2	Phytol
Secondary	2499.6	Salicin
Secondary	2549	Salicin
Secondary	2235.5	T-sinapinic acid
Unidentified metabolite	1068.5	Unknown 1
Unidentified metabolite	1073.1	Unknown 2
Unidentified metabolite	1091.9	Unknown 3
Unidentified metabolite	1179.6	Unknown 4
Unidentified metabolite	1183.2	Unknown 5
Unidentified metabolite	1184.4	Unknown 6
Unidentified metabolite	1193.7	Unknown 7
Unidentified metabolite	1330.8	Unknown 8
Unidentified metabolite	1332.5	Unknown 9
Unidentified metabolite	1347.1	Unknown 10
Unidentified metabolite	1359.1	Unknown 11
Unidentified metabolite	1367.9	Unknown 12
Unidentified metabolite	1418.6	Unknown 13
Unidentified metabolite	1432.3	Unknown 14
Unidentified metabolite	1440	Unknown 15
Unidentified metabolite	1441.4	Unknown 16
Unidentified metabolite	1479.3	Unknown 17

Compound class	RI	Compound ID
Unidentified metabolite	1534	Unknown 18
Unidentified metabolite	1553.4	Unknown 19
Unidentified metabolite	1555	Unknown 20
Unidentified metabolite	1584.6	Unknown 21
Unidentified metabolite	1587.7	Unknown 22
Unidentified metabolite	1617.6	Unknown 23
Unidentified metabolite	1631	Unknown 24
Unidentified metabolite	1631.9	Unknown 25
Unidentified metabolite	1650.7	Unknown 26
Unidentified metabolite	1651.9	Unknown 27
Unidentified metabolite	1662.7	Unknown 28
Unidentified metabolite	1665.4	Unknown 29
Unidentified metabolite	1668.1	Unknown 30
Unidentified metabolite	1716.3	Unknown 31
Unidentified metabolite	1742.3	Unknown 32
Unidentified metabolite	1752	Unknown 33
Unidentified metabolite	1768.8	Unknown 34
Unidentified metabolite	1783.9	Unknown 35
Unidentified metabolite	1837.3	Unknown 36
Unidentified metabolite	1861.1	Unknown 37
Unidentified metabolite	1892.1	Unknown 38
Unidentified metabolite	1918	Unknown 39
Unidentified metabolite	1922	Unknown 40
Unidentified metabolite	1937.4	Unknown 41
Unidentified metabolite	1938.7	Unknown 42
Unidentified metabolite	1941.1	Unknown 43
Unidentified metabolite	1946	Unknown 44
Unidentified metabolite	1979.5	Unknown 45
Unidentified metabolite	2020	Unknown 46
Unidentified metabolite	2029.2	Unknown 47
Unidentified metabolite	2055.7	Unknown 48
Unidentified metabolite	2073.7	Unknown 49
Unidentified metabolite	2083.1	Unknown 50
Unidentified metabolite	2159	Unknown 51
Unidentified metabolite	2160.8	Unknown 52
Unidentified metabolite	2169.1	Unknown 53
Unidentified metabolite	2195.5	Unknown 54
Unidentified metabolite	2258.6	Unknown 55
Unidentified metabolite	2263.2	Unknown 56

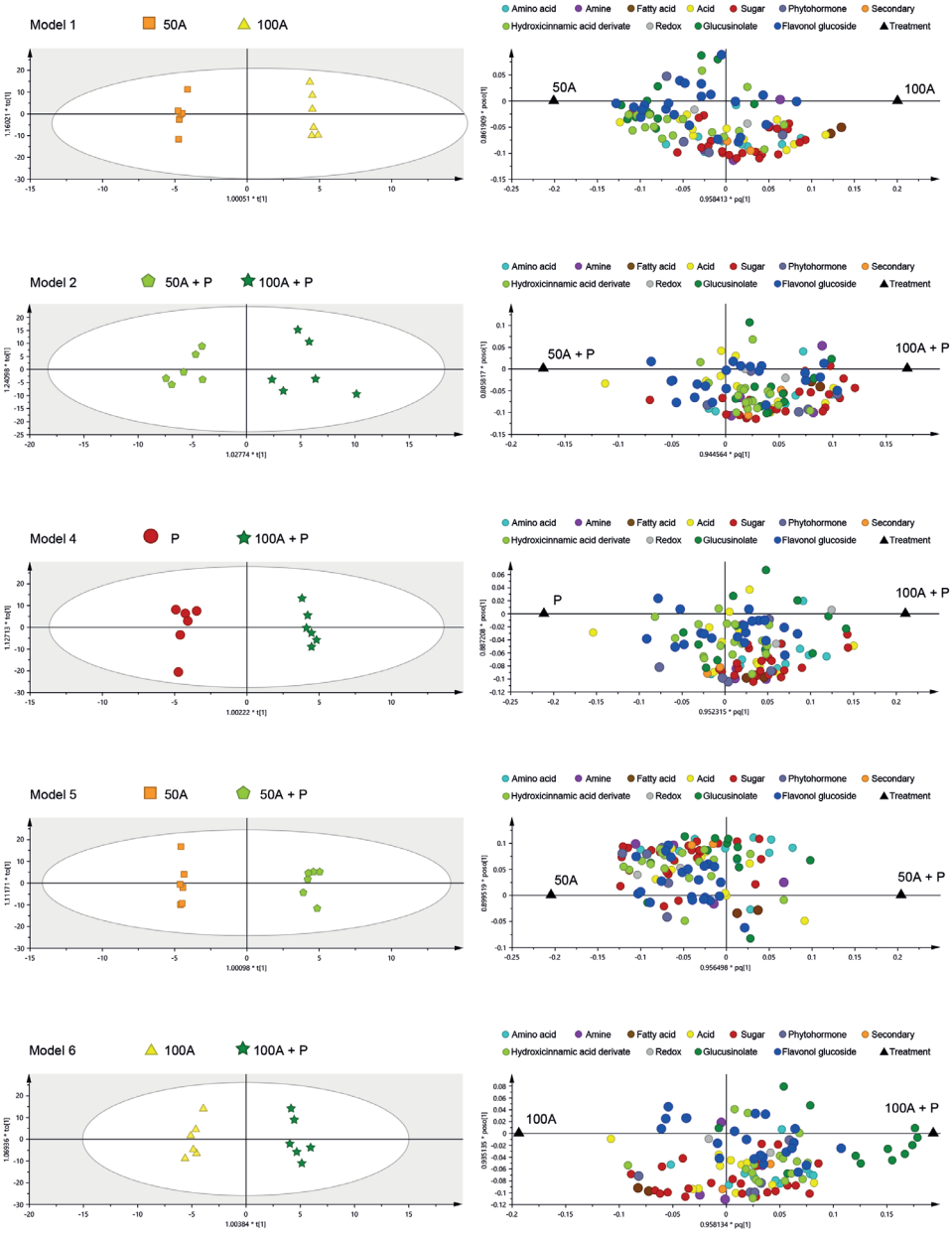
Compound class	RI	Compound ID
Unidentified metabolite	2285.4	Unknown 57
Unidentified metabolite	2288.8	Unknown 58
Unidentified metabolite	2347.9	Unknown 59
Unidentified metabolite	2351.3	Unknown 60
Unidentified metabolite	2356	Unknown 61
Unidentified metabolite	2359	Unknown 62
Unidentified metabolite	2367.5	Unknown 63
Unidentified metabolite	2417.9	Unknown 64
Unidentified metabolite	2422	Unknown 65
Unidentified metabolite	2452	Unknown 66
Unidentified metabolite	2464.9	Unknown 67
Unidentified metabolite	2473.1	Unknown 68
Unidentified metabolite	2476.8	Unknown 69
Unidentified metabolite	2522.6	Unknown 70
Unidentified metabolite	2560.1	Unknown 71
Unidentified metabolite	2568.4	Unknown 72
Unidentified metabolite	2694.5	Unknown 73
Unidentified metabolite	2705.9	Unknown 74
Unidentified metabolite	2730.9	Unknown 75
Unidentified metabolite	2764.8	Unknown 76
Unidentified metabolite	2784.2	Unknown 77
Unidentified metabolite	2793.1	Unknown 78
Unidentified metabolite	2804.4	Unknown 79
Unidentified metabolite	2808.9	Unknown 80
Unidentified metabolite	2822.9	Unknown 81
Unidentified metabolite	2829.1	Unknown 82
Unidentified metabolite	2913.4	Unknown 83
Unidentified metabolite	2934.2	Unknown 84
Unidentified metabolite	2965.6	Unknown 85
Unidentified metabolite	3012.5	Unknown 86
Unidentified metabolite	3027	Unknown 87
Unidentified metabolite	3034.9	Unknown 88
Unidentified metabolite	3072.5	Unknown 89
Unidentified metabolite	3092.8	Unknown 90
Unidentified metabolite	3125.7	Unknown 91
Unidentified metabolite	3130.7	Unknown 92
Unidentified metabolite	3225.5	Unknown 93
Unidentified metabolite	3258.3	Unknown 94
Unidentified metabolite	3284.7	Unknown 95

Compound class	RI	Compound ID
Unidentified metabolite	3328.5	Unknown 96
Unidentified metabolite	3373.8	Unknown 97
Unidentified metabolite	3439.3	Unknown 98
Unidentified metabolite	3498.8	Unknown 99

Table S2. Identified and unknown metabolites from LC-qTOF-MS analysis. Compound class, retention time (Rt), detected mass charge ration (m/z, negative ionization mode [M-H]-) and compound identity are reported. Flavonol glucoside abbreviations: Is = Isorhamnetin, Km = Kaempferol, Qn = Quercetin.

Compound class	Rt (min)	[M-H]- m/z	Compound ID
Glucosinolates	0.98	358.027	Sinigrin
Glucosinolates	0.98	717.061	Sinigrin dimer
Glucosinolates	1.58	372.042	Gluconapin
Glucosinolates	0.36	420.075	Glucoerucin
Glucosinolates	0.75	422.02	Glucoiberin
Glucosinolates	2.42	388.065	Glucojiaputin
Glucosinolates	2.91	386.118	Glucobrassicanapin
Glucosinolates	2.45	447.052	Glucobrassicin
Glucosinolates	1.75	463.045	4-hydroxy-glucobrassicin
Glucosinolates	3.0	477.065	4-methoxy-glucobrassicin
Glucosinolates	3.3	477.065	Neoglucobrassicin
Glucosinolates	2.83	422.061	Gluconasturtin
Redox metabolism	1.32	611.145	Glutathione oxidized GSSG
Redox metabolism	0.73	306.076	Glutathione reduced GSH
Hydroxycinnamic acid derivatives	3.9	163,035	p-coumaric-acid
Hydroxycinnamic acid derivatives	2.36	325.093	p-coumaroyl-D-glucose
Hydroxycinnamic acid derivatives	1.66	339.072	Sinapoylmalic acid
Hydroxycinnamic acid derivatives	1.33	341.088	1-Caffeoyl-β-D-glucose
Hydroxycinnamic acid derivatives	2.38	341.088	1-Caffeoyl-β-D-glucose
Hydroxycinnamic acid derivatives	2.55	371.099	Hydroxyferuloylglucose
Hydroxycinnamic acid derivatives	2.95	385.115	1-O-sinapoylglucose
Hydroxycinnamic acid derivatives	3.8	591.175	1-2-disinapoylglucoside
Hydroxycinnamic acid derivatives	3.6	723.215	Sinapoylferuloylgentiobiose
Hydroxycinnamic acid derivatives	3.43	739.209	Sinapoylhydroxyferuloylgentiobiose
Hydroxycinnamic acid derivatives	3.3	725.194	Dihydroxyferuloylgentiobiose
Hydroxycinnamic acid derivatives	3.6	753.225	Disinapoylgentiobiose

Compound class	Rt (min)	[M-H]- m/z	Compound ID
Hydroxycinnamic acid derivatives	3.13	753.225	Disinapoylgentiobiose
Hydroxycinnamic acid derivatives	3.8	929.273	Disinapoylferuloylgentiobiose
Hydroxycinnamic acid derivatives	3.61	945.268	Disinapoylhydroxyferuloylgentiobiose
Hydroxycinnamic acid derivatives	3.78	959.285	Trisinapoylgentiobiose
Flavonol glucosides	3.75	477.104	Is 3-glucoside
Flavonol glucosides	3.71	447.094	Km-7-glucoside
Flavonol glucosides	3.03	609.147	Km-3,7-diglucoside
Flavonol glucosides	2.7	771.199	Km-3-sophoroside-7-glucoside
Flavonol glucosides	3.47	815.205	Km 3-sinapoylsophoroside
Flavonol glucosides	3.05	947.247	Km 3-feruloylsophoroside-7-glucoside
Flavonol glucosides	3	977.256	Km 3-sinapoylsophoroside-7-glucoside
Flavonol glucosides	2.95	1139.309	Km 3-sinapoylsophorotrioside-7-glucoside
Flavonol glucosides	2.82	963.234	Km-3-hydroxyferuloylsophoroside-7-glucoside
Flavonol glucosides	3.08	917.236	Km-3-p-coumaroylsophoroside-7-glucoside
Flavonol glucosides	2.9	625.141	Qn-7-sophoroside
Flavonol glucosides	2.57	787.193	Qn 3-sophoroside-7-glucoside
Flavonol glucosides	2.58	787.193	Qn 3-sophoroside-7-glucoside
Flavonol glucosides	3.03	949.251	Qn-3-sophorotrioside-7-glucoside
Flavonol glucosides	2.77	949.225	Qn-3-caffeoylsophoroside-7-glucoside
Flavonol glucosides	2.87	933.231	Qn 3-p-coumaroylsophoroside-7-glucoside
Flavonol glucosides	2.75	993.253	Qn-3-sinapoylsophoroside-7-glucoside
Flavonol glucosides	2.71	1125.294	Qn 3-feruloylsophorotrioside-7-glucoside
Flavonol glucosides	2.53	979.245	Qn 3-hydrxyferuloylsophoroside-7-glucoside
Flavonol glucosides	2.78	1095.283	Qn 3-p-coumaroylsophorotrioside-7-glucoside
Flavonol glucosides	2.88	1155.305	Qn 3-sinapoylsophorotrioside-7-glucoside
Fatty acids	7.58	981.581	Unknown 111
Fatty acids	7.53	791.495	Unknown 222
Fatty acids	7.55	831.515	Unknown 333
Unidentified metabolite	0.43	440.083	Unknown 129
Unidentified metabolite	3	729.261	Unknown 148
Unidentified metabolite	0.43	729.235	Unknown 148 bis
Unidentified metabolite	2.95	499.105	Unknown 337
Unidentified metabolite	6.75	379.155	Unknown 356
Unidentified metabolite	6	349.145	Unknown 381
Unidentified metabolite	3.55	554.163	Unknown 554
Unidentified metabolite	3.55	553.163	Unknown 553
Unidentified metabolite	2.51	615.215	Unknown 615


Model 1
50A
100A
Model 2
50A + P
100A + P
Model 4
P
100A + P
Model 5
50A
50A + P
Model 6
100A
100A + P
Amino acid
Amine
Fatty acid
Acid
Sugar
Phytohormone
Secondary
Hydroxicinnamic acid derivate
Redox
Glucusinolate
Flavonol glucoside
Treatment

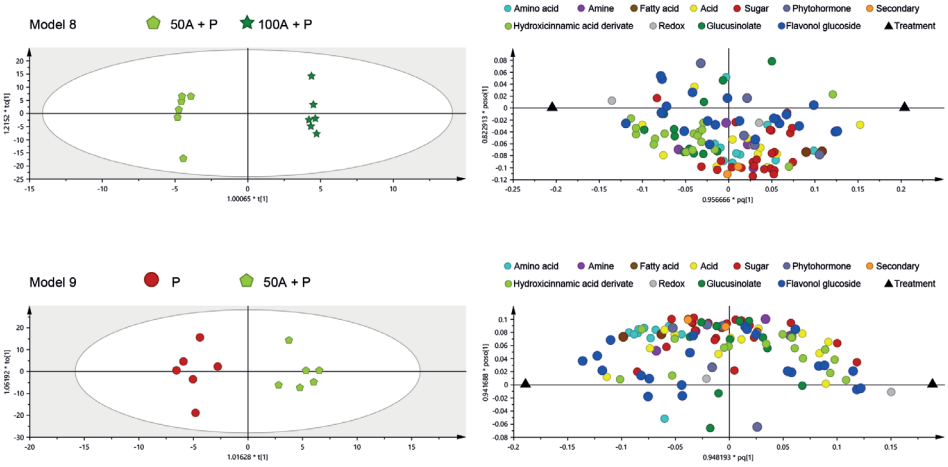
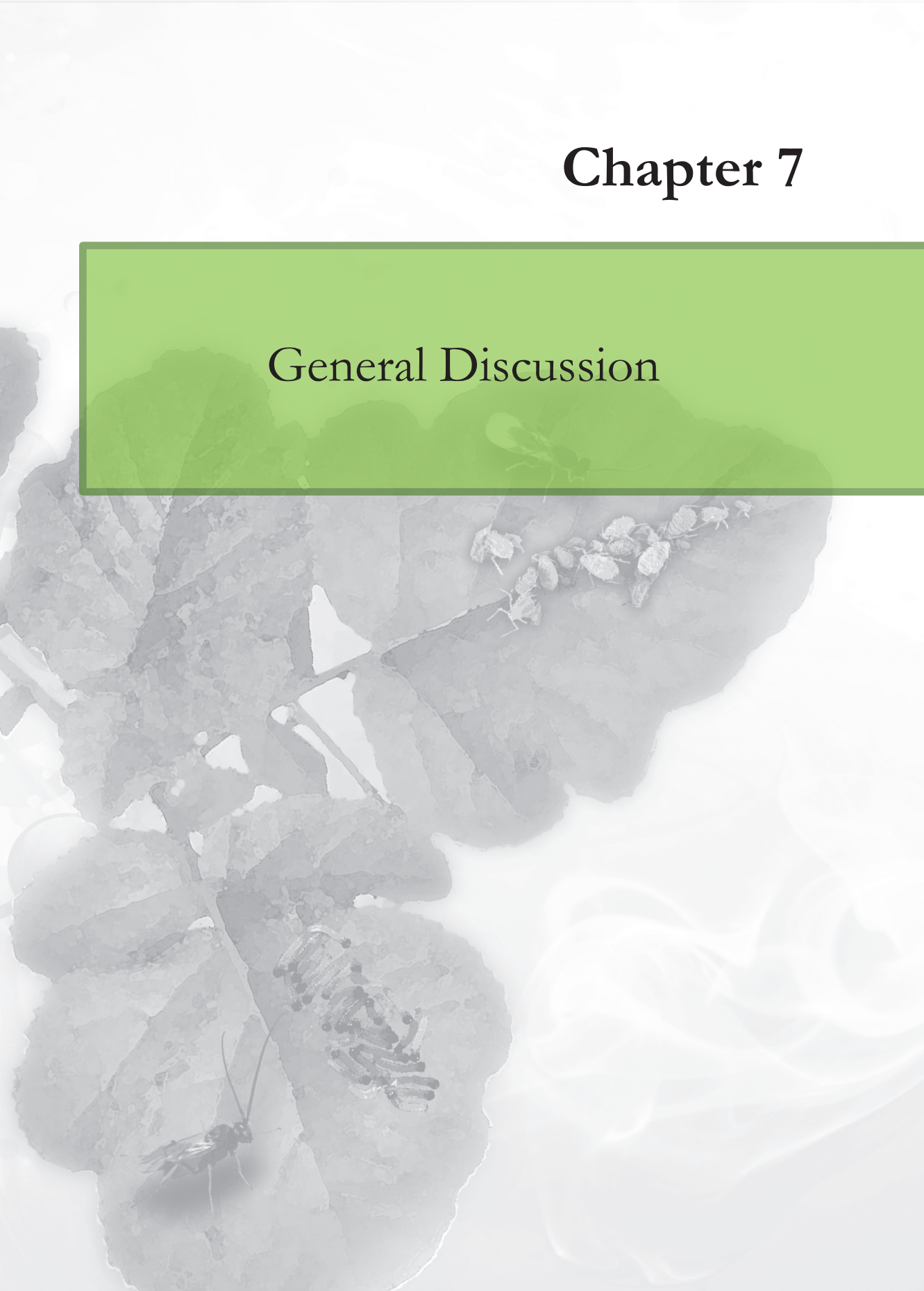


Figure S1. Multivariate analysis (OPLS-DA, with a) score plot and b) loading plot) of *B. nigra* leaf metabolic profiles, of the pairwise comparisons presented in Table 1, for which there was a separation between the two treatments. Model numbers refer to the numbers used in Table 1. Symbols in the score plot refer to healthy plants (C), plants infested with 50 *B. brassicae* aphids (50A), 100 aphids (100A), *P. brassicae* caterpillars (P), 50 aphids plus caterpillars (50A+P) or 100 aphids plus caterpillars (100A+P). The ellipse defines Hotelling's T2 confidence region (95 %).

Chapter 7

General Discussion



Introduction

As members of complex communities, plants in nature interact with a plethora of other organisms, and these interactions can have either beneficial or detrimental consequences for the plant. Pathogenic microbes and herbivorous insects represent two serious threats to plants. With about half of the estimated six million insect species thought to be herbivorous (Schoonhoven *et al.*, 2005), and a staggering diversity of phytopathogens (Agrios, 2005), plants need to implement effective defense mechanisms to overcome such adversity. Though plants are immobile, they are far from being passive and have developed, aside from their constitutive defenses, a wide arsenal of inducible defense strategies to deploy when under attack. These induced defenses can be direct, consisting of changes in plant chemistry or morphology that negatively influence the attacker's performance and behavior (Karban & Baldwin, 1997; Agrawal, 1998), or defenses can be indirect, by promoting the control activity of the natural enemies of the attackers. One example of indirect defense is the enhancement of the effectiveness of natural enemies of the herbivores to locate the infested plants (D'Alessandro & Turlings, 2006; Bruinsma & Dicke, 2008). This type of indirect defense is mediated by the plant's production and emission of volatile organic compounds (VOCs) in response to herbivore attack, with these volatiles being used as cues by foraging parasitoids or predators (Dicke, 1999; Clavijo McCormick *et al.*, 2012).

Yet in a typical natural situation, plants will often face multiple challengers simultaneously or sequentially, potentially having strong consequences for volatile-mediated foraging. In addition, while herbivore-induced plant volatiles (HIPVs) are well studied, only limited knowledge is available on the induction of plant volatiles under increasingly complex attack scenarios, and these studies are often restricted to investigating the effects of multiple insect herbivores (Rodriguez-Saona *et al.*, 2003; De Boer *et al.*, 2008; Zhang *et al.*, 2009; Bukovinszky *et al.*, 2012; Zhang *et al.*, 2013). Plant pathogens represent a major and omnipresent threat to plants, yet the effect of these on plant volatiles and their ensuing utilization by other members of plant communities, such as natural enemies of the insect herbivores, has been largely overlooked. In this thesis, I addressed this gap in our knowledge by evaluating the impact of combined biotic stresses on plant volatile induction and emission, on non-volatile metabolites, and the subsequent effect of the volatiles on the natural enemies of the attacking herbivores. The objectives of this thesis were to 1) determine if there was an impact of signaling pathway crosstalk on volatile production and the attraction of parasitoids, 2) investigate the impact of herbivore density on plant metabolism (volatile and non-volatile) and volatile-mediated

foraging behavior of different members of the third trophic level, and 3) explore if plant pathogen challenge exerts the same force as non-host herbivores in modifying volatile-mediated tritrophic interactions.

	Investigating the consequences of dual attack
Chapter 3:	Specificity of non-host attacker identity on induced volatile emissions and parasitoid foraging
Chapter 4:	Effects of aphid density during dual attack on plant volatile emissions and three parasitoid species
Chapter 5:	Plant pathogen virulence as a factor in volatile induction and wasp foraging
Chapter 6:	Aphid density and dual attack effects on the non-volatile plant metabolome

Figure 1. Overview of the experimental chapters contained in this thesis

Non-host effects: Is there a potential role for defense signaling crosstalk?

Volatile mediated foraging has been well studied, e.g. in the brassicaceae (Bruinsma *et al.*, 2009; Dicke *et al.*, 2009; Gols *et al.*, 2011), yielding detailed insight into the mechanisms and processes underlying tritrophic interactions between the plant, the attacking herbivore and the natural enemy (Turlings *et al.*, 1990; Unsicker *et al.*, 2009; Mumm & Dicke, 2010). However, in the context of multiple or dual attack it has often been questioned whether the presence of non-host attackers will negatively affect the foraging behavior of the natural enemies of the host attacker. It has long been assumed that due to defense signaling pathway crosstalk at the molecular level, which has amply been demonstrated in the literature and is discussed in Chapter 2, dual attack will exert a negative influence on tritrophic interactions by impeding successful host/prey location by natural enemies, via modifications in the induced volatile blend.

In Table 1, I present a (non-exhaustive) overview of the existing literature in aboveground study systems, which investigated the effects of dual attack on plant volatile emissions and/or the foraging behavior of natural enemies confronted with these volatiles. The overview focuses on studies where opposing signaling pathways are theoretically induced by the two attackers, on the basis of their feeding guilds, or from information gathered in scientific literature. From the studies in Table 1, it emerges that non-host attackers from a different feeding guild generally have a limited negative effect on parasitoid foraging preference: in the majority of studies the natural enemies do not discriminate between

dually infested and host-only infested plants (9/14 studies) and in only two studies were plants infested with hosts alone preferred. In general, volatile-mediated foraging behavior does not seem affected by the presence of non-host herbivores feeding on the same plant, and that theoretically induce an opposing defense signaling pathway. The notable exceptions to this are two studies by Zhang and collaborators (Zhang *et al.*, 2009; Zhang *et al.*, 2013), which used *Bemisi tabaci* whiteflies as the non-host inducer, and which had a strong negative effect on the attraction of two natural enemy species, a predator and a parasitoid.

The results presented in this thesis are largely consistent with the existing literature for *Cotesia glomerata*, in that parasitoid preference (in a host versus dual situation) will generally not be negatively affected by simultaneous non-host presence on host-infested plants. There was one exception; when plants had been infested with a high aphid density plus hosts I found a negative impact of non-host presence. *Cotesia glomerata* attraction to plant volatiles in response to dual attack has been extensively studied by de Rijk (2016). She found that various non-host species, their densities, or number of non-host species present on the plant had little negative effects on foraging behavior of *C. glomerata*, though she had a different approach by comparing non-host infested plants to dually infested plants. These results, in combination with my own, and other work from our lab (e.g. Soler *et al.*, 2012b) show that C. *glomerata* is rather flexible in its use of volatile cues, or relies on other, non-volatile cues as well. The aphid parasitoid *Diaeretiella rapae* was also unaffected by non-host presence, supporting previous work with this species (Agbogba & Powell, 2007), though unexpectedly I found strong negative effects of increasing host density: as host density increased, volatiles from these plants became less attractive to the wasps. Of the three parasitoid species that I investigated, the egg parasitoid *Trichogramma brassicae,* was the most strongly negatively affected by non-host presence, though in this case there was theoretically dual induction of the same defense signaling pathway (Kusnierczyk *et al.*, 2008; Bruessow *et al.*, 2010).

From the perspective of the volatiles induced during dual herbivory, in the literature surveyed in Table 1, the effects of dual attack are difficult to predict. In most cases the effects on blend composition are quantitative rather than qualitative, which is what I found in all the studies where I conducted headspace analyses. Generally, I found little effect of the initial attackers on the caterpillar-induced volatile blend. Attacker-specific effects could be seen in the volatile blend in the case of single attack, but when *P. brassicae* caterpillars were subsequently introduced onto the plant, dual-infestation treatments induced blends that resembled each other and were indistinguishable from the volatile blend induced by caterpillars alone, though phytopathogen challenge in Chapter 5 led to

stronger differentiation of the blends compared to dual infestation with aphids and eggs in Chapters 3 and 4. Aphid-infestation or egg deposition triggers changes in the plant on a smaller scale, likely due to the minimal damage they cause compared to the heavy damage inflicted by feeding caterpillars, and the effects of contrasting feeding guild is known to impact plant volatiles (Rowen & Kaplan, 2016), with leaf chewers inducing volatiles much more strongly than sap feeders. This same pattern could also be seen in the non-volatile metabolome profile of plants induced by aphids and caterpillars (Chapter 6), though in this case, more subtle effects of aphid infestation were also detected in the dual-attack treatments.

When delving deeper into the plant-mediated interactions between two attacker species, my results show that by manipulating certain variables within the same study system, parasitoid foraging decisions can be significantly altered. Chapters 3 and 4 both included dual attack by non-host aphids, yet while there was no effect of aphid density on the foraging behavior of *C.glomerata* in Chapter 3, an effect was present in Chapter 4. Crucially, this discrepancy was due to one minor change in the experimental set-up: the relative positioning of the herbivores on the plant. In Chapter 3 the caterpillars were positioned on the leaf directly above the aphid-infested leaf, while in Chapter 4 both herbivores infested the same leaf. An interference effect of aphids on caterpillar leaf consumption rate was ruled out by measuring the area of leaf tissue consumed on healthy or aphid-infested plants, and therefore the effect was expected to be due to changes in leaf chemistry. The leaf metabolome analysis revealed that while aphids did have a significant effect on the metabolome of the local, infested leaf, the systemic adjacent leaf was relatively unaffected by aphid infestation, suggesting that aphid-induced changes in the plant remain largely contained to the infested leaf, which has been found in previous studies on aphid-induced changes in plants (Moran & Thompson, 2001; De Vos *et al.*, 2005). This supports the discrepancies between the experiments in Chapters 3 and 4, as aphid infestation would be expected to more strongly impact caterpillar-induced plant volatiles, and subsequent utilization of these volatiles as cues by foraging parasitoids, when they are present on the same leaf as the aphids.

I also identified two other factors which very strongly influenced the outcome of dual attack on volatile-mediated foraging. Firstly, by modifying the density of the attacking non-host herbivore, both the chemical and behavioral responses were affected, with wasp foraging preference even being inverted by increasing density. Secondly, plant pathogens were found to have a much larger impact on volatiles and volatile-mediated foraging than was anticipated. The effect of these two important factors will be discussed in greater detail in the following sections.

Table 1. Overview of studies testing the effects of dual attack on induced plant volatiles and/or volatile-mediated foraging behavior of naïve natural enemies, when the two attackers theoretically induce opposing defense signaling pathways, focusing on natural enemy responses to host-infested plants against dually infested plants. (*) indicates the focal attacker: either the host/prey species of the studied natural enemy, or, in the case of studies investigating only volatiles, the attacker when effects of a second attacker on its induced volatile blend was measured. (+) indicates that the attacker was a plant pathogen, (↓) decrease, (↑) increase, (⊘) no difference.

Plant species	Jasmonic acid inducing attacker	Salicylic acid inducing attacker	Natural enemy	Volatile preference of natural enemy		Dual attack effects on VOCs compared to focal attacker	Notes	Reference
				Control vs Dual	Host/prey vs Dual			
Asteraceae								
Solidago altissima	*Heliothis virescens* *	*Eurosta solidaginis*				↓ total emissions		Tooker *et al.* 2008
S. altissima	*H. virescens* *	*Gnorimoschema gallaesolidaginis*				⊘, except for 1 compound		Tooker *et al.* 2008
S. altissima	*H. virescæns* *	*Philaenus spumarius*				⊘		Tooker *et al.* 2008
Brassicaceae								
Arabidopsis thaliana	*Plutella xylostella* *	*Bemisia. tabaci*	*Diadegma semiclausum*		Host	All treatments different from one another		Zhang *et al.* 2013
Brassica nigra	*Pieris brassicae* (larvae)	*Pieris brassicae* (eggs)*	*Trichogramma brassicae*	Dual	Equal	Same compounds but different ratios		Cusumano *et al.* 2015
B. nigra	*Spodoptera exigua*	*P. brassicae* (eggs)*	*T. brassicae*	Dual	Equal	Same compounds but different ratios		Cusumano *et al.* 2015
B. nigra	*P. brassicae* (larvae)	*P. brassicae* (eggs)*	*T. evanescens*	Equal	Equal	Same compounds but different ratios		Cusumano *et al.* 2015
B. nigra	*S. exigua*	*P. brassicae* (eggs)*	*T. evanescens*	Equal	Equal	Same compounds but different ratios		Cusumano *et al.* 2015

Plant species	Jasmonic acid inducing attacker	Salicylic acid inducing attacker	Natural enemy	Volatile preference of natural enemy		Dual attack effects on VOCs compared to focal attacker	Notes	Reference
				Control vs Dual	Host/prey vs Dual			
B. nigra	*P. brassicae* (larvae)*	*P. brassicae* (eggs)	*Cotesia glomerata*		Dual (and equal at 2 other time points)	OPLS-DA shows separation, dual = 1enhanced compound and 2 suppressed.	Tested 6 larval ages (1h to 72h)	Pashalidou *et al.* 2015
Brassica oleracea	*P. brassicae**	*Brevicoryne brassicae*	*C. glomerata*		Equal	Quantitative differences, but blends are similar	2 aphid densities (15 and 50)	Soler *et al.* 2012
B. oleracea	*P. xylostella*	*Myzus persicae* *	*Diaeretiella rapae*		Equal			Agbogba and Powell, 2007
B. oleracea	*P. brassicae**	*B. brassicae*	*C. glomerata*		Dual vs non-host : dual			De Rijk *et al.* 2016b
B. oleracea	*P. brassicae**	*M. persicae*	*C. glomerata*		Dual vs non-host : dual			De Rijk *et al.* 2016b
Fabaceae								
Arachis hypogaea	*S. exigua* *	*Scerotium rolfsii*+	*C. marginiventris*		Dual			Cardoza *et al.* 2003
A. hypogaea	*S.exigua**	*S. rolfsii* +				Additive effect of both individual blends		Cardoza *et al.* 2002
Phaseolus lunatus	*Tetranychus urticae**	*B. tabaci*	*Phytoseiulus persimilis*		Host	↓ of one important compound	↓ in (*E*)-β-ocimene	Zhang *et al.* 2009
Vicia faba	*S. exigua**	*Acyrthosiphon pisum*				↓ emissions of many compounds		Schwartzberg *et al.* 2011
Malvaceae								
Gossypium hirsutum	*S. exigua**	*B. tabaci*				↓ total emissions		Rodriguez-Saona *et al.* 2003

Plant species	Jasmonic acid inducing attacker	Salicylic acid inducing attacker	Natural enemy	Volatile preference of natural enemy		Dual attack effects on VOCs compared to focal attacker	Notes	Reference
				Control vs Dual	Host/prey vs Dual			
Solanacaeae								
Capsicum annuum	*T. urticae**	*M. persicae**	*Macrolophus caliginosus*		Dual	↑ emissions, 2 unique compounds	Both are prey species	Moayeri *et al.* 2007
Datura wrightii	*Lema daturophila* *	*Tupiocoris notatus*				↑ total emissions		Hare *et al.* 2011
Poaceae								
Zea mays	*S. littoralis* *	*Euscelidius variegatus*	*C. marginiventris*	Dual	Equal	⊘	4 arm olfactometer	Erb *et al.* 2010
Z. mays	*S. littoralis* *	*Setosphaeria turcica* +	*Microplitis rufiventris*		Equal	↓ in total volatiles	6 arm olfactormeter. Pathogen alone not attractive	Rostas *et al.* 2006
Z. mays	*S. littoralis* *	*S.turcica* +	*C. marginiventris*		Equal	↓ in total volatiles	Pathogen alone not attractive	Rostas *et al.* 2006

How many individuals attack is just as important as who attacks

One of the keys aspects emerging from this thesis is that herbivore density can be strongly influential to both foraging behavior of parasitoid wasps, and to the plant metabolome. In Chapters 3 and 4, I showed that foraging of three parasitoid wasp species was affected by *B. brassicae* aphid density, even though the aphids represent a non-host for *C. glomerata* and *T. brassicae*, and are the host of *D. rapae*. Aphid density also had an effect on the volatile metabolome of infested plants, but it was generally weaker and visible only when comparing the treatments where aphids were the only attacker. Interestingly, the highest and lowest densities grouped together, separate from the intermediate density. While intriguing, I could find no functional explanation for the non-linearity of the volatile profiles. In addition, the density effects observed in the volatiles did not match with the *D. rapae* behavioral data, which showed a linear effect of aphid density, yet an opposite, non-linear effect was present in the volatile data. When I investigated the effects of aphid density on the non-volatile metabolome, in Chapter 6, aphid density effects were also present, though also weaker than the caterpillar-induced effects. The two aphid densities had different effects on leaf metabolomic profiles, but it was not possible to determine if there was also non-linearity in the leaf metabolome, as the third, and lowest, density was not included in this set of experiments. Thus, considering all these data together, I conclude that while there are attacker species-specific effects on plant chemistry, attacker density will further modulate the plants' defense response to a given species both in single and dual attack scenarios, and can in turn modify the interpretation of the chemical cues used by parasitoid wasps.

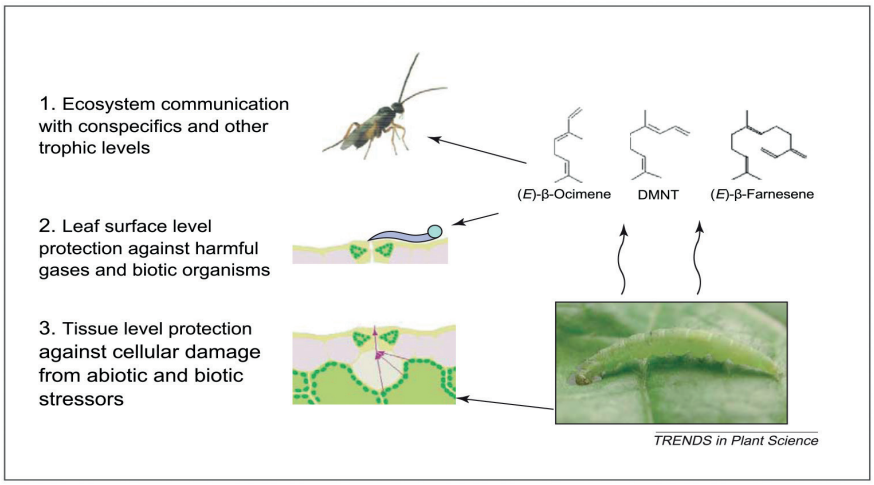


Figure 2. A model of different functional levels of induced volatiles (adapted from Holopainen 2004)

Herbivore populations are dynamic, and can change rapidly over time. This is especially true for aphids, which have very high reproductive rates that lead to exponential population growth in a relatively short time frame, when under ideal conditions. Research on attack by multiple herbivores is typically investigated with a fixed herbivore density. While this is understandable, as it can be necessary to limit the complexity of oftentimes already complex experiments and research questions, further effort should be made to address the influence of herbivore density on plant-herbivore and plant-natural enemy interactions. During attack by a single herbivore, herbivore density can strongly affect plant volatile emissions (Shiojiri *et al.*, 2010; Cai *et al.*, 2014; Truong *et al.*, 2014) and also its metabolome (Ossipov *et al.*, 2014), so when these density effects are coupled to the effects of dual herbivore attack, important interactive effects should be expected. Other studies have included density effects (Zhang *et al.*, 2009; Kroes *et al.*, 2015; De Rijk *et al.*, 2016a), and show that during dual herbivore attack, interference in plant defenses generally occurs at the higher tested density. In lima bean, *Bemisia tabaci* whiteflies interfered in a density-dependent manner with the plants' indirect defense against spider mites, with a positive correlation between whitefly density and interference with attraction (Zhang *et al.*, 2009). However, de Rijk *et al.* (2016a) found limited effects of four co-infesting non-host species on wasp foraging, with only one non-host species modifying landing preference of *C. glomerata* when it was present at a high density, though this was studied in a non-host environment, e.g. both plants were non-host infested, with one having hosts as well, contrary to other studies and my own. At the gene expression level, Kroes *et al.* (2015) showed interference of *B. brassicae* with induced defense against co-attacking *Plutella xylostella* caterpillars, with this effect present only at the highest tested aphid density, and also showed that body mass of caterpillars feeding on these plants was positively affected at the lower density, but negatively affected by the higher aphid density. Thus, when considering my own results along with these other studies, it strongly suggests that there is a herbivore population gradient along which plant defenses are induced, with higher herbivore densities leading to different defense induction dynamics than do lower densities. It appears imperative for studies to include different herbivore densities in order to have a better picture of the complexities of defense induction and their subsequent effects on other trophic levels.

However, one shortcoming of these studies, and my own included, is that they all fail to take into account natural population dynamics: herbivore colonies generally originate from a small number of individuals and build up to the thereafter studied 'low' or 'high' densities over the course of several days to several weeks. Defense induction could be significantly different between a plant confronted by a sudden and unnaturally

high herbivore infestation, and a plant exposed to a natural and gradually increasing infestation. However, to my knowledge there are no gene expression data or otherwise to lend credence to this hypothesis. Such knowledge would be important to have a better understanding of the mechanisms of plant defense in a realistic and natural scenario. For instance, it has been shown for caterpillar herbivory that it is important to consider plant defense mechanisms from the moment of oviposition, and not just at the moment that the freshly emerged caterpillars start feeding on the plant (Pashalidou *et al.*, 2014; Hilker & Fatouros, 2015). However, in terms of natural density increase effects on induced volatiles and parasitoid foraging, some data indicate that a density increase can have species-specific negative effects on volatile-mediated foraging. Li *et al.* (2016) tested two aphid pre-infestation durations on parasitoid foraging, and found that *Diadegma semiclausum* wasps preferred volatiles of dually infested cabbage plants over those of host-only infested plants at 7 days infestation (approx. 160 aphids) but this preference switched when tested at 14 days of aphid infestation (approx. 430 aphids). The other tested parasitoid species, *Microplitis mediator* was overall more attracted to dually infested plants regardless of the duration of aphid infestation. The results with *D. semiclausum* have a strong parallel with the results I obtained with *C. glomerata* in Chapter 3, in that there appears to be a preference switch as aphid density increases, even in the case of natural population growth.

Pathogens influence herbivore-induced plant volatiles

One of the most striking results of this thesis is that pathogen challenge can have a strong effect on induced plant volatiles (Chapters 3 and 5), leading to stronger treatment-dependent effects on the volatile blends than the non-host herbivorous attackers that were tested (Chapters 3 and 4). This research takes the field of multiple attack and tritrophic interactions into a relatively new and unexplored direction that would benefit from greater attention. Typically, the effects of pathogen challenge in relation to herbivory have been fairly well studied in the context of plant-mediated effects on herbivore or pathogen performance, or changes to the defense signaling pathways at the molecular level (Cui *et al.*, 2002; De Vos *et al.*, 2006; Stout *et al.*, 2006; Lazebnik *et al.*, 2014). In terms of research on pathogen-induced volatiles, some work has been done on the induction of pathogen-specific compounds, mostly in the context of early disease detection in crops or their inhibitory effects on future pathogen colonization, and even less has been done in terms of effects on tritrophic interactions (Cardoza *et al.*, 2003b; Rostás *et al.*, 2006). But given the ubiquitous presence of plant pathogens in nature, if we

are to have a more complete and realistic view of the effects of dual attack on volatile-mediated foraging, we must make more efforts to include plant pathogens, as they can have a profound impact on volatile-mediated foraging, as demonstrated in this thesis.

I found that pathogen-induced plant volatiles were highly attractive to the larval parasitoid *C. glomerata*, regardless of whether the pathogen was virulent or avirulent on *B. nigra*, and regardless of the presence of host caterpillars on the plant. Dual challenge with a pathogen and caterpillars even enhanced the attractiveness of the volatiles to *C. glomerata* compared to a plant infested only with caterpillars. Remarkably, when the effects of the virulent strain were tested at several time points post-infection (C. Ponzio, unpublished data), the dually-challenged plants were highly attractive at the first (24 hour) time point, well before the onset of symptoms. This preference continued through to the last time point (14 days), when the pathogen had spread systemically through the plant. *P. brassicae* performance data indicated that pupal mass was not affected by plants infected with either pathogen (C. Ponzio, unpublished data), so wasps would likely not be gaining a fitness benefit by preferring to parasitize hosts on pathogen-infected plants. Thus, pathogen infection appears to only increase the apparency of the plants to the parasitoids. Even more striking was the high attractiveness of pathogen-induced volatiles when tested against healthy plants, which was recorded for both the virulent and avirulent strains that were tested. This is not without precedent, as previous work in another system has shown that microbial elicitors can induce volatile emissions bearing strong resemblance to HIPVs (Leitner *et al.*, 2008). These results raise a valid question: why would a pathogen-challenged plant emit volatiles which then risk misinforming foraging parasitoids, and attracting them to a host-free plant? The answer to this may depend on whether the problem is seen from the plant's or parasitoid's perspective, and we need to consider the different possible functions of plant volatiles.

Typically, induced plant volatiles have been considered to be a cue in the interaction of plant and natural enemy, conferring protection, indirectly, to plants from ravenous herbivores. The role of plant volatiles in attracting carnivorous enemies of insect herbivores has been extensively studied in the last 35 years (for extensive reviews see Dicke & van Loon, 2000; Mumm & Dicke, 2010; Hare, 2011). However, given the high frequency of multiple attack in natural situations, it can be expected that parasitoids have evolved the flexibility to cope with large variability in the emitted volatiles, with this variability either due to multiple attackers, or due to interspecific variation across the different host-plant species that the parasitoids may forage on. Volatile cues are not the only cues that wasps can rely on to locate their hosts; visual cues or host products such as frass can also supply valuable information (van Alphen *et al.*, 2003; Colazza

et al., 2014). Response to volatiles is only the first phase of foraging, and wasps may rapidly move on to another plant once they have noted the absence of hosts (De Rijk *et al.*, 2016b). It is undeniable that induced plant volatiles play a central role in mediating interactions between plants and insects, and with other community members, but given the diversity of plant volatile compounds and the many physiological processes which volatile induction can stem from, plant volatiles can serve a large variety of functions, of which attraction of natural enemies of attacking herbivores is just one of them (Dicke & Baldwin, 2010).

Entomologists studying volatile-mediated foraging behavior by parasitoids may be focusing too much on this one role, as important as it may be, and lose sight of the multi-functionality of plant VOC emissions, and their more general role in plant defense (Holopainen, 2004; Holopainen & Gershenzon, 2010) (Fig. 2). Leaf volatiles play an important role in plant communication with other members of its community. They can have repellent effects against herbivores (Kessler & Baldwin, 2001), but can also serve as cues that insect herbivores can use to locate suitable host plants for feeding (Bolter *et al.*, 1997). Volatiles from plants under attack also play a role in plant-plant communication, and prime the defenses of unattacked neighboring plants (Howe & Jander, 2008). Several common vegetative volatiles, also induced by insect herbivory, have been shown to have protective effects against pathogen infection (Croft *et al.*, 1993; Shulaev *et al.*, 1997; Cardoza *et al.*, 2002). Many VOCs have key roles in protecting plants from the damaging effects of abiotic stress, for example by conferring a level of protection against the oxidative effects of heat or ozone stress (Peñuelas & Llusià, 2003; Holopainen, 2004).

Plant metabolites: considering compounds individually or as a mixture?

In the 35 years since the seminal paper by Price et al (1980) arguing the importance of considering tritrophic interactions, a wealth of studies have been produced demonstrating the use of HIPVs by predators and parasitoids to locate their herbivorous victims. While the knowledge on the mechanisms and complexity of volatile induction is ever increasing, our understanding of how natural enemies use these volatile cues has not kept pace with these developments. While we have a lot of evidence that parasitoids use volatile cues, we have rarely established a direct link between parasitoid behavior and induced plant volatile profiles, and despite years of ongoing research we are only slightly closer to understanding what it is in the volatiles that help orient different parasitoids species. Electroantennogram-based assays have allowed to determine which individual compounds are detected and elicit a response in the insect sensory system (Smid *et al.*,

2002; van Tol & Visser, 2002; Bruce *et al.*, 2005), which is valuable information, but this does not give clues as to the relative importance of each compound within a volatile mixture, or even their general relevance across a wider range of plant species, in the case of parasitoid species whose dietary breadth includes multiple plant species. Indeed, while Smid et al. (2002) identified a number of compounds emitted by *Brassica oleracea* which elicit an electroantennogram response in *C. glomerata*, I did not find any of these compounds in any in headspace analyses which I conducted, yet wasps still located host-infested plants. While this does not mean that these previously identified compounds are not important, it does reveal several things: 1) important compounds in one plant species may not be universal in the Brassicaceae and so *C. glomerata* utilizes a wide range of compounds in its foraging decisions and 2) despite *C. glomerata* being extensively studied, especially in our lab, we still do not know what volatiles are important for this wasp, and this is true for other parasitoid species as well, such as the also well-studied *C. marginiventris*. In contrast, for acarine predators the bioacitivity of individual plant volatiles and mixtures of compounds in attraction to prey-infested plants and discrimination between prey-infested and non-prey-infested plants has been demonstrated (Dicke *et al.*, 1990; De Boer & Dicke, 2004; De Boer *et al.*, 2004).

In Chapters 3, 4 and 5, I show that *B. nigra* plants emit the same compounds across all the applied induction treatments, and that induced plants emit, with one or two exceptions, the same compounds as healthy control plants. This indicates that, for this plant species at least, foraging wasps are not relying on the simple binary presence or absence of a compound, but are relying on overall mixture characteristics, as shown for foraging predators as well (De Boer *et al.*, 2004). In a large number of plant-insect interactions, overall blend composition is crucial. This has been amply demonstrated for herbivorous insects, showing a role for a specific ratio or combination of certain compounds (Visser & Avé, 1978; Natale *et al.*, 2003; Bruce *et al.*, 2005). For natural enemies, while individual key foraging compounds have been identified for some species (De Boer *et al.*, 2004; Ibrahim *et al.*, 2005; Rasmann *et al.*, 2005; Halitschke *et al.*, 2008; Zhang *et al.*, 2009), for many other species it is expected that they rely on a mixture of many compounds rather than one or two specific compounds, and sometimes these mixtures must be present against a specific odor background in order to become attractive (De Boer *et al.*, 2004; Mumm & Hilker, 2005).

Chapter 4 shows that there is a nonlinear response to aphid density in the overall volatile mixture, yet if each of the compounds had been considered and analyzed separately, there were very few affected in an aphid-density specific manner, likely in part due to the inherently large amount of natural variation present in *B. nigra* populations. As a

predominantly outcrossing plant species, *B. nigra* has larger genetic variability and lower population differentiation than found in selfing species, and so induction of individual metabolites can strongly vary between plants. However, even when treatment-induced differences are minor for an individual compound, if this happens over a large number of compounds, all these small differences can add up to a strong effect at the overall mixture level, which is the level at which insect perception take place. This is where multivariate data analysis shows a strong advantage: multivariate modeling focuses on the differences between whole volatile blends, and can highlight the relative importance of a compound, or a group of compounds, in the odor mixture. This approach also gives equal importance to the minor blend constituents that may have a significant impact on foraging insects (Clavijo McCormick *et al.*, 2014) and on the volatile signature as a whole. Until the precise cues used by foraging wasps can be deciphered, analyzing the whole blend characteristics rather than individual compounds may more closely approximate the 'analysis' of the volatiles which the insects make, and indicate which blend characteristics may be most relevant to wasp foraging. However, this approach is not without its limitations, as seen in this thesis. While it allows analysis of a whole blend, we do not know if this is what the insects are using; they may be relying on a smaller, bioactive set within the whole blend, or they may be responding to compounds present in such trace amounts that they may be beneath the current analytical detectability thresholds (Gouinguené *et al.*, 2005).

In my research I also showed that for the non-volatile metabolome, it is also important to first consider the metabolome as a whole, rather than look for effects on individual compounds in response to herbivory in order to reveal global patterns of change. This approach showed that some metabolite pools, such as sugars or glucosinolates, were affected in a treatment-specific way, and then aphid-density can further impact those pools with specific sugars or glucosinolates more strongly induced at the higher density, e.g. trehalose or fructose for the sugars. By having a more global view of the changes induced by herbivory, it also facilitates the identification of strongly influential metabolites that may vary in a highly treatment-specific manner as well. Thus I could identify that redox-system related glutathione metabolites (GSH and the oxidized GSSG form) responded in a species-specific and density-specific manner, with GSH concentrations increasing with increasing aphid density, indicating that plants were under higher stress.

Concluding remarks and further perspectives

The results of this research project represent another step forward in our understanding

of the complex interactions that exist between plants, herbivores and parasitoids, providing new insight in how dual biotic attack will modify not just induced plant volatiles and the behavior of the parasitoids which depend on these for host-location, but also reconfigure the metabolism of the attacked plant. While the volatile cues that are used by the larval parasitoid *C. glomerata* are relatively robust and resist interference by non-host attackers present on the plant whether they be herbivorous or microbial (Chapter 3 & 5), changes in relative positioning of the attackers on the plant and changes in aphid density may strongly affect the behavior of parasitoids for which aphids represent a non-host, as well as for a parasitoid of the aphids (Chapter 3). By analyzing the whole metabolome of plants subjected to dual attack with different aphid densities, it was revealed that dual attack leads to treatment-specific effects on both primary and secondary metabolites (Chapter 6), and this was a unique step forward in understanding how dual attack affects plant responses at the subcellular level. However, insect herbivores are not alone in their capacity to impact multi-trophic interactions, and this research also stressed the importance of including biotic attackers suck as plant pathogens, which can have profound effects on induced plant volatiles and parasitoid foraging behavior, even in the case of a strain that *B. nigra* is resistant to.

Over the course of the past 15 years, an increasing number of studies have focused on the effects of dual attack on multi-trophic interactions. These studies have yielded an exciting new perspective on how natural enemies respond to volatile cues in more complex situations, and provided insight in how plants modulate their defense responses when facing multiple attackers. However, there are still many challenges in this field. One of the major findings of this thesis was that pathogen challenge, whether or not the plants were susceptible or resistant to the strains studied, can have very strong effects on volatile emission and parasitoid foraging, even more so than non-host herbivores that were also included in this thesis. Although a plant's reactions to insect herbivores and plant pathogens have by and large been considered separate and distinct from one another, the defense responses induced by both types of attackers overlap considerably, while also displaying attacker specificity (Stout *et al.*, 1999; De Vos *et al.*, 2005). It is important for research on plant defenses to strive towards greater integration of plant-pathogen and plant-insect interactions, which would provide a more comprehensive overview of the modulation of plant defenses. Pathogen effects on herbivore fitness, and vice versa have been studied, but we are still lacking detailed information on their interactions at the plant subcellular level, and even more so at the community level, where natural enemy responses have been largely ignored. Having detailed knowledge of plant-insect-pathogen interactions at multiple levels of biological integration will be crucial for better understanding how plants

modulate defense responses to different groups of attacking organisms, and provide a more realistic view of the ecology of tritrophic interactions.

Future work on multiple attack should seek to move towards more realism, by working with more ecologically relevant study systems. Part of the difficulty in detecting trends within the body of literature on dual attack at the molecular or ecological level is that in a large number of cases, the study systems do not naturally occur. Use of cultivated plants which strongly differ from their wild ancestors is common, as is mixing plant and herbivore species that would be unlikely to co-occur in natural situation (Harvey *et al.*, 2015). Ecological realism can be pushed further, by focusing more on the impact of factors that can affect tritrophic interactions, rather than further increasing complexity by including more attackers. Attacker density or their spatial and/or temporal separation on the plants during dual attack appear to exert stronger influence on tritrophic interactions than species identity, and so merit further investigation. A next step would be to include more natural experimental field set-ups, in conjunction to the traditional laboratory-based assays. While this controlled approach has yielded valuable information and insights, if we are to truly understand the ecological and evolutionary context of plant volatiles as foraging cues, it is necessary to step out of the laboratory and take research out in the field, in the plants' and insects' natural environment. Plants out in the field may produce very different volatile blends (Kigathi *et al.*, 2009), though behavioral responses of foraging insects seem robust and persist out in the field (Poelman *et al.*, 2009; Lucas-Barbosa *et al.*, 2013).

Stress-induced modifications to plant chemistry can result in important changes in the biological community that is associated with plants and so can have important consequences for plant fitness. By providing a detailed overview on how dual attack with both herbivores and plant pathogens affects plant chemistry and parasitoid foraging, this thesis contributes important and fundamental insight into the plant-mediated effects of dual attack on parasitoid foraging. By further integrating pathogen- and herbivore-plant interactions in both molecular ecology and community ecology, this will increase the complexity of research to be done, but also provide exciting new insights into the modulation of plant defenses.

Acknowledgements

I thank Rieta Gols and Marcel Dicke for their constructive comments on earlier versions of this chapter.

References

Agbogba BC, Powell W. 2007. Effect of the presence of a nonhost herbivore on the response of the aphid parasitoid *Diaeretiella rapae* to host-infested cabbage plants. *Journal of Chemical Ecology* **33**(12): 2229-2235.

Agrawal A. 1998. Induced responses to herbivory and increased plant performance. *Science* **279**: 1201-1202.

Agrios GN. 2005. *Plant Pathology*. San Diego: Elsevier Academic Press.

Alborn HT, Turlings TCJ, Jones TH, Stenhagen G, Loughrin JH, Tumlinson JH. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* **276**(5314): 945-949.

Algarra Alarcon A, Lazazzara V, Cappellin L, Bianchedi PL, Schuhmacher R, Wohlfahrt G, Pertot I, Biasioli F, Perazzolli M. 2015. Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation in vitro. *Journal of Mass Spectrometry* **50**(8): 1013-1022.

Arany AM, de Jong TJ, Kim HK, van Dam NM, Choi YH, Verpoorte R, van der Meijden E. 2008. Glucosinolates and other metabolites in the leaves of *Arabidopsis thaliana* from natural populations and their effects on a generalist and a specialist herbivore. *Chemoecology* **18**(2): 65-71.

Arimura G, Matsui K, Takabayashi J. 2009. Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. *Plant and Cell Physiology* **50**(5): 911-923.

Attaran E, Rostás M, Zeier J. 2008. *Pseudomonas syringae* elicits emission of the terpenoid (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4. *Molecular Plant-Microbe Interactions* **21**(11): 1482-1497.

Baldwin I, Schmelz E, Ohnmeiss T. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and comes. *Journal of Chemical Ecology* **20**(8): 2139-2157.

Ballhorn DJ. 2011. Constraints of simultaneous resistance to a fungal pathogen and an insect herbivore in Lima Bean (*Phaseolus lunatus* L.). *Journal of Chemical Ecology* **37**(2): 141-144.

Blande JD, Pickett JA, Poppy GM. 2004. Attack rate and success of the parasitoid *Diaeretiella rapae* on specialist and generalist feeding aphids. *Journal of Chemical Ecology* **30**(9): 1781-1795.

Blande JD, Pickett JA, Poppy GM. 2007. A comparison of semiochemically mediated interactions involving specialist and generalist *Brassica*-feeding aphids and the braconid parasitoid *Diaeretiella rapae*. *Journal of Chemical Ecology* **33**(4): 767-779.

Bleeker PM, Diergaarde PJ, Ament K, Guerra J, Weidner M, Schütz S, de Both MTJ, Haring MA, Schuurink RC. 2009. The role of specific tomato volatiles in tomato-whitefly interaction. *Plant Physiology* **151**(2): 925-935.

Bolter CJ, Dicke M, Van Loon JJA, Visser JH, Posthumus MA. 1997. Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *Journal of Chemical Ecology* **23**(4): 1003-1023.

Bonaventure G, VanDoorn A, Baldwin IT. 2011. Herbivore-associated elicitors: FAC signaling and metabolism. *Trends in Plant Science* **16**(6): 294-299.

Bosque-Pérez NA, Eigenbrode SD. 2011. The influence of virus-induced changes in plants on aphid vectors: Insights from luteovirus pathosystems. *Virus Research* **159**(2): 201-205.

Bradburne RP, Mithen R. 2000. Glucosinolate genetics and the attraction of the aphid parasitoid *Diaeretiella rapae* to Brassica. *Proceedings of the Royal Society of London B: Biological Sciences* **267**(1438): 89-95.

Brown G, Prochaska G, Hildebrand D, Nordin G, Jackson D. 1995. Green leaf volatiles inhibit conidial germination of the entomopathogen *Pandora neoaphidis* (Entomopthorales: Entomophthoraceae). *Environmental Entomology* **24**(6): 1637-1643.

Bruce TJA, Pickett JA. 2011. Perception of plant volatile blends by herbivorous insects – finding the right mix. *Phytochemistry* **72**(13): 1605-1611.

Bruce TJA, Wadhams LJ, Woodcock CM. 2005. Insect host location: a volatile situation. *Trends in Plant Science* **10**(6): 269-274.

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P. 2010. Insect eggs suppress plant defence against chewing herbivores. *The Plant Journal* **62**(5): 876-885.

Bruinsma M, Dicke M 2008. Herbivore-induced indirect defense: from induction mechanisms to community ecology. In: Schaller A ed. *Induced Plant Resistance to Herbivory*: Springer Netherlands, 31-60.

Bruinsma M, Posthumus MA, Mumm R, Mueller MJ, van Loon JJA, Dicke M. 2009. Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *Journal of Experimental Botany* **60**(9): 2575-2587.

Bukovinszky T, Poelman EH, Kamp A, Hemerik L, Prekatsakis G, Dicke M. 2012. Plants under multiple herbivory: consequences for parasitoid search behaviour and foraging efficiency. *Animal Behaviour* **83**(2): 501-509.

Bukovinszky T, van Veen FJF, Jongema Y, Dicke M. 2008. Direct and indirect effects of resource quality on food web structure. *Science* **319**(5864): 804-807.

Cai X-M, Sun X-L, Dong W-X, Wang G-C, Chen Z-M. 2014. Herbivore species, infestation time, and herbivore density affect induced volatiles in tea plants. *Chemoecology* **24**(1): 1-14.

Cardé RT, Millar JG. 2004. *Advances in insect chemical ecology*: Cambridge University Press Cambridge.

Cardoza Y, Tumlinson J. 2006. Compatible and incompatible *Xanthomonas* infections differentially affect herbivore induced volatile emission by pepper plants. *Journal of Chemical Ecology* **32**(8): 1755-1768.

Cardoza YJ, Alborn HT, Tumlinson JH. 2002. In vivo volatile emissions from peanut plants induced by simultaneous fungal infection and insect damage. *Journal of Chemical Ecology* **28**(1): 161-174.

Cardoza YJ, Lait CG, Schmelz EA, Huang J, Tumlinson JH. 2003a. Fungus-induced biochemical changes in peanut plants and their effect on development of beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) larvae. *Environmental Entomology* **32**(1): 220-228.

Cardoza YJ, Teal PEA, Tumlinson JH. 2003b. Effect of peanut plant fungal infection on oviposition preference by *Spodoptera exigua* and on host-searching behavior by *Cotesia marginiventris*. *Environmental Entomology* **32**(5): 970-976.

Chew F 1988. Biological effects of glucosinolates. In: Cutler, H. G. (ed.) Biologically Active Natural Products-Potential Use in Agriculture, *ACS symposium series*: Oxford University Press. 155-181.

Chung C-L, Longfellow J, Walsh E, Kerdieh Z, Van Esbroeck G, Balint-Kurti P, Nelson R. 2010. Resistance loci affecting distinct stages of fungal pathogenesis: use of introgression lines for QTL mapping and characterization in the maize - *Setosphaeria turcica* pathosystem. *BMC Plant Biology* **10**(1): 103.

Clarke DB. 2010. Glucosinolates, structures and analysis in food. *Analytical Methods* **2**(4): 310-325.

Clavijo McCormick A, Gershenzon J, Unsicker SB. 2014. Little peaks with big effects: establishing the role of minor plant volatiles in plant–insect interactions. *Plant, Cell & Environment* **37**(8): 1836-1844.

Clavijo McCormick A, Unsicker SB, Gershenzon J. 2012. The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. *Trends in Plant Science* **17**(5): 303-310.

Colazza S, Cusumano A, Lo Giudice D, Peri E. 2014. Chemo-orientation responses in hymenopteran parasitoids induced by substrate-borne semiochemicals. *BioControl* **59**(1): 1-17.

Colazza S, Fucarino A, Peri E, Salerno G, Conti E, Bin F. 2004a. Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *Journal of Experimental Biology* **207**(1): 47-53.

Colazza S, McElfresh JS, Millar J. 2004b. Identification of volatile synomones, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalis*. *Journal of Chemical Ecology* **30**(5): 945-964.

Conti E, Zadra C, Salerno G, Leombruni B, Volpe D, Frati F, Marucchini C, Bin F. 2008. Changes in the volatile profile of *Brassica oleracea* due to feeding and opposition by *Murgantia histrionica* (Heteroptera: Pentatomidae). *European Journal of Entomology* **105**(5): 839-847.

Croft K, Juttner F, Slusarenko AJ. 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. *Plant Physiology* **101**(1): 13-24.

Cui J, Jander G, Racki LR, Kim PD, Pierce NE, Ausubel FM. 2002. Signals involved in Arabidopsis resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. *Plant Physiology* **129**(2): 551-564.

D'Alessandro M, Turlings TCJ. 2006. Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. *Analyst* **131**(1): 24-32.

De Boer J, Hordijk C, Posthumus M, Dicke M. 2008. Prey and non-prey arthropods sharing a host plant: effects on induced volatile emission and predator attraction. *Journal of Chemical Ecology* **34**(3): 281-290.

De Boer JG, Dicke M. 2004. The role of methyl salicylate in prey searching behavior of the predatory mite *Phytoseiulus persimilis*. *Journal of Chemical Ecology* **30**(2): 255-271.

De Boer JG, Posthumus MA, Dicke M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. *Journal of Chemical Ecology* **30**(11): 2215-2230.

De Moraes C, Lewis W, Pare P, Alborn H, Tumlinson J. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* **393**(6685): 570 - 573.

De Oliveira CF, Long EY, Finke DL. 2014. A negative effect of a pathogen on its vector? A plant pathogen increases the vulnerability of its vector to attack by natural enemies. *Oecologia* **174**(4): 1169-1177.

De Rijk M. 2016. *Foraging behaviour of parasitoids in multi-herbivore communities*. PhD thesis, Wageningen University Wageningen, The Netherlands.

De Rijk M, Dicke M, Poelman EH. 2013. Foraging behaviour by parasitoids in multiherbivore communities. *Animal Behaviour* **85**(6): 1517-1528.

De Rijk M, van der Loo JAH, Engel B, Dicke M, Poelman EH. 2016a. Density- and trait- mediated indirect interactions alter host foraging behaviour of parasitoids without altering foraging efficiency. *(submitted)*.

De Rijk M, Yang D, Engel B, Dicke M, Poelman EH. 2016b. Feeding guild of non-host community members affects host-foraging efficiency of a parasitic wasp. *Ecology (in press)*.

De Vos M, Van Oosten V, Van Poecke R, Van Pelt J, Pozo M, Mueller M, Buchala A, Metraux J, Van Loon L, Dicke M, et al. 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Molecular Plant-Microbe Interactions* **18**(9): 923 - 937.

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC, Pieterse CMJ. 2006. Herbivore-induced resistance against microbial pathogens in Arabidopsis. *Plant Physiology* **142**(1): 352-363.

Degen T, Dillmann C, Marion-Poll F, Turlings TCJ. 2004. High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiology* **135**(4): 1928-1938.

Delphia C, Mescher M, De Moraes C. 2007. Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defenses on host-plant selection by thrips. *Journal of Chemical Ecology* **33**(5): 997-1012.

Denno R, McClure M, Ott J. 1995. Interspecific interactions in phytophagous insects: competition reexamined and resurrected. *Annual Review of Entomology* **40**(1): 297-331.

Dicke M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging

carnivorous arthropods? *Entomologia Experimentalis et Applicata* **91**(1): 131-142.

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

Dicke M, Grostal P. 2001. Chemical detection of natural enemies by arthropods: an ecological perspective. *Annual Review of Ecology and Systematics* **32**(2001): 1-23.

Dicke M, Sabelis M, de Jong M. 1988. Analysis of prey preference in phytoseiid mites by using an olfactometer, predation models and electrophoresis. *Experimental & Applied Acarology* **5**(3-4): 225-241.

Dicke M, Van Baarlen P, Wessels R, Dijkman H. 1993. Herbivory induces systemic production of plant volatiles that attract predators of the herbivore: Extraction of endogenous elicitor. *Journal of Chemical Ecology* **19**(3): 581-599.

Dicke M, Van Beek TA, Posthumus MA, Ben Dom N, Van Bokhoven H, De Groot A. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions Involvement of host plant in its production. *Journal of Chemical Ecology* **16**(2): 381-396.

Dicke M, van Loon JJA. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomologia Experimentalis et Applicata* **97**(3): 237-249.

Dicke M, van Loon JJA, Soler R. 2009. Chemical complexity of volatiles from plants induced by multiple attack. *Nature Chemical Biology* **5**(5): 317-324.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT. 2009. Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiology* **150**(3): 1576-1586.

Dötterl S, Jürgens A, Wolfe L, Biere A. 2009. Disease status and population origin effects on floral scent: potential consequences for oviposition and fruit predation in a complex interaction between a plant, fungus, and noctuid moth. *Journal of Chemical Ecology* **35**(3): 307-319.

Doughty KJ, Blight MM, Bock CH, Fieldsend JK, Pickett JA. 1996. Release of alkenyl isothiocyanates and other volatiles from *Brassica rapa* seedlings during infection by *Alternaria brassicae*. *Phytochemistry* **43**(2): 371-374.

Dudareva N, Negre F, Nagegowda DA, Orlova I. 2006. Plant volatiles: recent advances and future perspectives. *Critical Reviews in Plant Sciences* **25**(5): 417-440.

Dudareva N, Pichersky E, Gershenzon J. 2004. Biochemistry of plant volatiles. *Plant Physiology* **135**(4): 1893-1902.

Eigenbrode SD, Ding H, Shiel P, Berger PH. 2002. Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera: Aphididae). *Proceedings of the Royal Society of London. Series B: Biological Sciences* **269**(1490): 455-460.

El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, Daayf F, Vojnov A, Bouarab K. 2011. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell Online* **23**(6): 2405-2421.

Engelberth J, Alborn HT, Schmelz EA, Tumlinson JH. 2004. Airborne signals prime plants against insect herbivore attack. *Proceedings of the National Academy of Sciences of the United States of America* **101**(6): 1781-1785.

Engelberth J, Koch T, Schüler G, Bachmann N, Rechtenbach J, Boland W. 2001. Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in Lima bean. *Plant Physiology* **125**(1): 369-377.

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

Erb M, Foresti N, Turlings T. 2010. A tritrophic signal that attracts parasitoids to host-damaged plants

withstands disruption by non-host herbivores. *BMC Plant Biology* **10**(1): 247.

Erb M, Köllner TG, Degenhardt J, Zwahlen C, Hibbard BE, Turlings TCJ. 2011a. The role of abscisic acid and water stress in root herbivore-induced leaf resistance. *New Phytologist* **189**(1): 308-320.

Erb M, Robert CAM, Hibbard BE, Turlings TCJ. 2011b. Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology* **99**(1): 7-15.

Eriksson L, Kettaneh-Wold N, Trygg J, Wikström C, Wold S. 2006. *Multi-and megavariate data analysis: Part I: Basic principles and applications*. Umeå, Sweden: Umetrics AB.

Fahey JW, Zalcmann AT, Talalay P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **56**(1): 5-51.

Fatouros NE, Dicke M, Mumm R, Meiners T, Hilker M. 2008. Foraging behavior of egg parasitoids exploiting chemical information. *Behavioral Ecology* **19**(3): 677-689.

Fatouros NE, Lucas-Barbosa D, Weldegergis BT, Pashalidou FG, van Loon JJA, Dicke M, Harvey JA, Gols R, Huigens ME. 2012. Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. *Plos One* **7**(8): e43607.

Fei M, Gols R, Harvey JA. 2014. Seasonal phenology of interactions involving short-lived annual plants, a multivoltine herbivore and its endoparasitoid wasp. *Journal of Animal Ecology* **83**(1): 234-244.

Fereres A, Moreno A. 2009. Behavioural aspects influencing plant virus transmission by homopteran insects. *Virus Research* **141**(2): 158-168.

Fernandez O, Béthencourt L, Quero A, Sangwan RS, Clément C. 2010. Trehalose and plant stress responses: friend or foe? *Trends in Plant Science* **15**(7): 409-417.

Frost CJ, Mescher MC, Carlson JE, De Moraes CM. 2008. Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology* **146**(3): 818-824.

Geervliet J, Vet L, Dicke M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomologia Experimentalis et Applicata* **73**(3): 289-297.

Geervliet JBF, Ariëns S, Dicke M, Vet LEM. 1998. Long-distance assessment of patch profitability through volatile infochemicals by the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae). *Biological Control* **11**(2): 113-121.

Geervliet JBF, Posthumus MA, Vet LEM, Dicke M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *Journal of Chemical Ecology* **23**(12): 2935-2954.

Geervliet JBF, Vet LEM, Dicke M. 1996. Innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) to volatiles from different plant-herbivore complexes. *Journal of Insect Behavior* **9**(4): 525-538.

Ghanta S, Bhattacharyya D, Sinha R, Banerjee A, Chattopadhyay S. 2011. *Nicotiana tabacum* overexpressing γ-ECS exhibits biotic stress tolerance likely through NPR1-dependent salicylic acid-mediated pathway. *Planta* **233**(5): 895-910.

Giordanengo P, Brunissen L, Rusterucci C, Vincent C, van Bel A, Dinant S, Girousse C, Faucher M, Bonnemain J-L. 2010. Compatible plant-aphid interactions: how aphids manipulate plant responses. *Comptes Rendus Biologies* **333**(6–7): 516-523.

Girling RD, Stewart-Jones A, Dherbecourt J, Staley JT, Wright DJ, Poppy GM. 2011. Parasitoids select plants more heavily infested with their caterpillar hosts: a new approach to aid interpretation of plant headspace volatiles. *Proceedings of the Royal Society B: Biological Sciences* **278**(1718): 2646-2653.

Giron D, Frago E, Pieterse CMJ, Dicke M. 2013. Cytokinins as key regulators in plant-microbe-insect interactions: connecting plant growth and defence. *Functional Ecology* **27**(3): 599–609.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* **43**: 205-227.

Gols R, Bullock J, Dicke M, Bukovinszky T, Harvey J. 2011. Smelling the wood from the trees: non-linear parasitoid responses to volatile attractants produced by wild and cultivated cabbage. *Journal of Chemical Ecology* **37**(8): 795-807.

Gols R, Harvey J. 2009. Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. *Phytochemistry Reviews* **8**(1): 187-206.

Gols R, Roosjen M, Dijkman H, Dicke M. 2003. Induction of direct and indirect plant responses by jasmonic acid, low spider mite densities, or a combination of jasmonic acid treatment and spider mite infestation. *Journal of Chemical Ecology* **29**(12): 2651-2666.

Gols R, Van Dam NM, Raaijmakers CE, Dicke M, Harvey JA. 2009. Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? *Oikos* **118**(5): 733-742.

Gols R, Veenemans C, Potting RPJ, Smid HM, Dicke M, Harvey JA, Bukovinszky T. 2012. Variation in the specificity of plant volatiles and their use by a specialist and a generalist parasitoid. *Animal Behaviour* **83**(5): 1231-1242.

Gols R, Witjes LMA, Van Loon JJA, Posthumus MA, Dicke M, Harvey JA. 2008. The effect of direct and indirect defenses in two wild brassicaceous plant species on a specialist herbivore and its gregarious endoparasitoid. *Entomologia Experimentalis et Applicata* **128**(1): 99-108.

Gouinguené S, Pickett JA, Wadhams LJ, Birkett MA, Turlings TCJ. 2005. Antennal electrophysiological responses of three parasitic wasps to caterpillar-Induced volatiles from maize (*Zea mays mays*), Cotton (*Gossypium herbaceum*), and Cowpea (*Vigna unguiculata*). *Journal of Chemical Ecology* **31**(5): 1023-1038.

Gouinguené SP, Turlings TCJ. 2002. The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiology* **129**(3): 1296-1307.

Grant MR, Jones JDG. 2009. Hormone (dis)harmony moulds plant health and disease. *Science* **324**(5928): 750-752.

Halitschke R, Gase K, Hui D, Schmidt DD, Baldwin IT. 2003. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. *Plant Physiology* **131**(4): 1894-1902.

Halitschke R, Stenberg JA, Kessler D, Kessler A, Baldwin IT. 2008. Shared signals –'alarm calls' from plants increase apparency to herbivores and their enemies in nature. *Ecology Letters* **11**(1): 24-34.

Halkier BA, Gershenzon J. 2006. Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* **57**(1): 303-333.

Hare J, Sun J. 2011. Production of induced volatiles by *Datura wrightii* in response to damage by insects: effect of herbivore species and time. *Journal of Chemical Ecology* **37**(7): 751-764.

Hare JD. 2011. Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annual Review of Entomology* **56**(1): 161-180.

Harvey JA, Malcicka M, Ellers J. 2015. Integrating more biological and ecological realism into studies of multitrophic interactions. *Ecological Entomology* **40**(4): 349-352.

Heil M. 2008. Indirect defence via tritrophic interactions. *New Phytologist* **178**(1): 41 - 61.

Heil M, Karban R. 2010. Explaining evolution of plant communication by airborne signals. *Trends in Ecology & Evolution* **25**(3): 137-144.

Hildebrand JG. 1995. Analysis of chemical signals by nervous systems. *Proceedings of the National Academy of Sciences* **92**(1): 67-74.

Hilker M, Fatouros NE. 2015. Plant responses to insect egg deposition. *Annual Review of Entomology* **60**(1): 493-515.

Hilker M, Kobs C, Varama M, Schrank K. 2002. Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *Journal of Experimental Biology* **205**(4): 455.

Hodge S, Powell G. 2008. Complex interactions between a plant pathogen and insect parasitoid via the shared vector-host: consequences for host plant infection. *Oecologia* **157**(3): 387-397.

Hodge S, Ward J, Beale M, Bennett M, Mansfield J, Powell G. 2013. Aphid-induced accumulation of trehalose in *Arabidopsis thaliana* is systemic and dependent upon aphid density. *Planta* **237**(4): 1057-1064.

Holopainen JK. 2004. Multiple functions of inducible plant volatiles. *Trends in Plant Science* **9**(11): 529-533.

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

Hopkins RJ, van Dam NM, van Loon JJA. 2009. Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annual Review of Entomology* **54**: 57-83.

Horiuchi J-I, Arimura G-I, Ozawa R, Shimoda T, Takabayashi J, Nishioka T. 2003. A comparison of the responses of *Tetranychus urticae* (Acari: Tetranychidae) and *Phytoseiulus persimilis* (Acari: Phytoseiidae) to volatiles emitted from lima bean leaves with different levels of damage made by *T. urticae* or *Spodoptera exigua* (Lepidoptera: Noctuidae). *Applied Entomology and Zoology* **38**(1): 109-116.

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

Huang J, Cardoza YJ, Schmelz EA, Raina R, Engelberth J, Tumlinson JH. 2003. Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae*. *Planta* **217**(5): 767-775.

Huang J, Schmelz E, Alborn H, Engelberth J, Tumlinson J. 2005. Phytohormones mediate volatile emissions during the interaction of compatible and incompatible pathogens: the role of ethylene in *Pseudomonas syringae* infected tobacco. *Journal of Chemical Ecology* **31**(3): 439-459.

Iason GR, Dicke M, Hartley SE. 2012. *The ecology of plant secondary metabolites: from genes to global processes*. Cambridge: Cambridge University Press.

Ibrahim M, Nissinen A, Holopainen J. 2005. Response of *Plutella xylostella* and its parasitoid *Cotesia plutellae* to volatile compounds. *Journal of Chemical Ecology* **31**(9): 1969-1984.

Jansen R. 2011. Detection of diseased plants by analysis of volatile organic compound emissions. *Annual Review of Phytopathology* **49**(1): 157-174.

Johne AB, Weissbecker B, Schütz S. 2008. Approaching risk assessment of complex disease development in horse chestnut trees: a chemical ecologist's perspective. *Journal of Applied Entomology* **132**(5): 349-359.

Kaplan I, Denno RF. 2007. Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. *Ecology Letters* **10**(10): 977-994.

Kappers I, Hoogerbrugge H, Bouwmeester H, Dicke M. 2011. Variation in herbivory-induced volatiles among cucumber (*Cucumis sativus* L.) varieties has consequences for the attraction of carnivorous natural enemies. *Journal of Chemical Ecology* **37**(2): 150-160.

Karban R, Baldwin I. 1997. *Induced responses to herbivory*. Chicago Chicago University Press.

Kazan K, Manners JM. 2008. Jasmonate signaling: toward an integrated view. *Plant Physiology* **146**(4): 1459-1468.

Kersten B, Ghirardo A, Schnitzler J-P, Kanawati B, Schmitt-Kopplin P, Fladung M, Schroeder H. 2013. Integrated transcriptomics and metabolomics decipher differences in the resistance of pedunculate oak to the herbivore *Tortrix viridana* L. *BMC Genomics* **14**(1): 737.

Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* **291**(5511): 2141-2144.

Kessler A, Baldwin IT. 2002. Plant responses to insect herbivory: the emerging molecular analysis. *Annual*

Review of Plant Biology **53**(1): 299-328.

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

Keurentjes JJB, Angenent GC, Dicke M, Santos VAPMD, Molenaar J, van der Putten WH, de Ruiter PC, Struik PC, Thomma BPHJ. 2011. Redefining plant systems biology: from cell to ecosystem. *Trends in Plant Science* **16**(4): 183-190.

Khaling E, Papazian S, Poelman EH, Holopainen JK, Albrectsen BR, Blande JD. 2015. Ozone affects growth and development of *Pieris brassicae* on the wild host plant *Brassica nigra*. *Environmental Pollution* **199**(0): 119-129.

Kigathi R, Unsicker S, Reichelt M, Kesselmeier J, Gershenzon J, Weisser W. 2009. Emission of volatile organic compounds after herbivory from *Trifolium pratense* (L.) under laboratory and field conditions. *Journal of Chemical Ecology* **35**(11): 1335-1348.

Kim JH, Jander G. 2007. *Myzus persicae* (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. *The Plant Journal* **49**(6): 1008-1019.

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC, Pieterse CMJ. 2008. Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiology* **147**(3): 1358-1368.

Koornneef A, Pieterse CMJ. 2008. Cross talk in defense signaling. *Plant Physiology* **146**(3): 839-844.

Kroes A, van Loon JJA, Dicke M. 2015. Density-dependent interference of aphids with caterpillar-induced defenses in Arabidopsis: involvement of phytohormones and transcription factors. *Plant and Cell Physiology* **56**(1): 98-106.

Kusnierczyk A, Winge PER, JØRstad TS, Troczynska J, Rossiter JT, Bones AM. 2008. Towards global understanding of plant defence against aphids – timing and dynamics of early Arabidopsis defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. *Plant, Cell & Environment* **31**(8): 1097-1115.

Kutyniok M, Müller C. 2012. Crosstalk between above- and belowground herbivores is mediated by minute metabolic responses of the host *Arabidopsis thaliana*. *Journal of Experimental Botany* **63**(17): 6199-6210.

Lazebnik J, Frago E, Dicke M, van Loon JA. 2014. Phytohormone mediation of interactions between herbivores and plant pathogens. *Journal of Chemical Ecology* **40**(7): 730-741.

Leitner M, Boland W, Mithöfer A. 2005. Direct and indirect defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*. *New Phytologist* **167**(2): 597-606.

Leitner M, Kaiser R, Rasmussen MO, Driguez H, Boland W, Mithöfer A. 2008. Microbial oligosaccharides differentially induce volatiles and signalling components in *Medicago truncatula*. *Phytochemistry* **69**(10): 2029-2040.

Leon-Reyes A, Du Y, Koornneef A, Proietti S, Körbes AP, Memelink J, Pieterse CMJ, Ritsema T. 2010. Ethylene signaling renders the jasmonate response of *Arabidopsis* insensitive to future suppression by salicylic acid. *Molecular Plant-Microbe Interactions* **23**(2): 187-197.

Li Y, Weldegergis BT, Chamontri S, Dicke M, Gols R. 2016. Does aphid infestation interfere with plant indirect defence against lepidopteran caterpillars in wild cabbage? *(submitted)*.

Lin L-Z, Sun J, Chen P, Harnly J. 2011. UHPLC-PDA-ESI/HRMS/MS(n) analysis of anthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives in red mustard greens (*Brassica juncea* coss variety). *Journal of Agricultural and Food Chemistry* **59**(22): 12059-12072.

Liu X, Xiang W, Jiao X, Zhang Y, Xie W, Wu Q, Zhou X, Wang S. 2014. Effects of plant virus and its insect vector on *Encarsia formosa*, a biocontrol agent of whiteflies. *Scientific Reports* **4**.

Liu X, Zhang S, Whitworth RJ, Stuart JJ, Chen M-S. 2015. Unbalanced activation of glutathione metabolic pathways suggests potential involvement in plant defense against the gall midge *Mayetiola destructor*

in wheat. *Scientific Reports* **5**: 8092.

Loreto F, Schnitzler JP. 2010. Abiotic stresses and induced BVOCs. *Trends in Plant Science* **15**(3): 154-166.

Lucas-Barbosa D, van Loon JJA, Dicke M. 2011. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. *Phytochemistry* **72**(13): 1647-1654.

Lucas-Barbosa D, van Loon JJA, Gols R, van Beek TA, Dicke M. 2013. Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production. *Functional Ecology* **27**(1): 245-254.

Maeda T, Takabayashi J. 2001. Production of herbivore-induced plant volatiles and their attractiveness to *Phytoseius persimilis* (Acari: Phytoseiidae) with changes of *Tetranychus urticae* (Acari: Tetranychidae) density on a plant. *Applied Entomology and Zoology* **36**(1): 47-52.

Mann RS, Ali JG, Hermann SL, Tiwari S, Pelz-Stelinski KS, Alborn HT, Stelinski LL. 2012. Induced release of a plant-defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen. *PLoS Pathogens* **8**(3): e1002610.

Marti G, Erb M, Boccard J, Glauser G, Doyen GR, Villard N, Robert CAM, Turlings TCJ, Rudaz S, Wolfender J-L. 2013. Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. *Plant, Cell & Environment* **36**(3): 621-639.

Martini X, Pelz-Stelinski KS, Stelinski LL. 2014. Plant pathogen-induced volatiles attract parasitoids to increase parasitism of an insect vector. *Frontiers in Ecology and Evolution* **2**(8).

Mathur V, Tytgat TG, de Graaf R, Kalia V, Sankara Reddy A, Vet LM, van Dam N. 2013. Dealing with double trouble: consequences of single and double herbivory in *Brassica juncea*. *Chemoecology* **23**(2): 71-82.

Mattiacci L, Dicke M. 1995. Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. *Entomologia Experimentalis et Applicata* **76**(1): 37-48.

Mattiacci L, Dicke M, Posthumus M. 1994. Induction of parasitoid attracting synomone in brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *Journal of Chemical Ecology* **20**(9): 2229-2247.

Mattiacci L, Dicke M, Posthumus MA. 1995. β-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Sciences* **92**(6): 2036-2040.

Mauck KE, De Moraes CM, Mescher MC. 2010. Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proceedings of the National Academy of Sciences* **107**(8): 3600-3605.

Mayer C, Vilcinskas A, Gross J. 2008. Phytopathogen lures Its insect vector by altering host plant odor. *Journal of Chemical Ecology* **34**(8): 1045-1049.

McLeod G, Gries R, von Reuß SH, Rahe JE, McIntosh R, König WA, Gries G. 2005. The pathogen causing Dutch elm disease makes host trees attract insect vectors. *Proceedings of the Royal Society B: Biological Sciences* **272**(1580): 2499-2503.

Meiners T, Hilker M. 1997. Host location in *Oomyzus gallerucae* (Hymenoptera: Eulophidae), an egg parasitoid of the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae). *Oecologia* **112**(1): 87-93.

Mithöfer A, Boland W. 2008. Recognition of herbivory-associated molecular patterns. *Plant Physiology* **146**(3): 825-831.

Mithöfer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**(1): 431-450.

Moayeri HRS, Ashouri A, Poll L, Enkegaard A. 2007. Olfactory response of a predatory mirid to herbivore induced plant volatiles: multiple herbivory vs. single herbivory. *Journal of Applied Entomology* **131**(5): 326-332.

Moran PJ, Thompson GA. 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant

defense pathways. *Plant Physiology* **125**(2): 1074-1085.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113**(7): 935-944.

Moujahed R, Frati F, Cusumano A, Salerno G, Conti E, Peri E, Colazza S. 2014. Egg parasitoid attraction toward induced plant volatiles is disrupted by a non-host herbivore attacking above or belowground plant organs. *Frontiers in Plant Science* **5**: 601.

Mousavi SAR, Chauvin A, Pascaud F, Kellenberger S, Farmer EE. 2013. GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. *Nature* **500**(7463): 422-426.

Mumm R, Burow M, Bukovinszkine'Kiss G, Kazantzidou E, Wittstock U, Dicke M, Gershenzon J. 2008. Formation of simple nitriles upon glucosinolate hydrolysis affects direct and indirect defense against the specialist herbivore, *Pieris rapae*. *Journal of Chemical Ecology* **34**(10): 1311-1321.

Mumm R, Dicke M. 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. *Canadian Journal of Zoology* **88**(7): 628-667.

Mumm R, Hilker M. 2005. The significance of background odour for an egg parasitoid to detect plants with host eggs. *Chemical Senses* **30**(4): 337-343.

Mumm R, Schrank K, Wegener R, Schulz S, Hilker M. 2003. Chemical analysis of volatiles emitted by *Pinus sylvestris* after induction by insect oviposition. *Journal of Chemical Ecology* **29**(5): 1235-1252.

Mur LAJ, Kenton P, Atzorn R, Miersch O, Wasternack C. 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology* **140**(1): 249-262.

Natale D, Mattiacci L, Hern A, Pasqualini E, Dorn S. 2003. Response of female *Cydia molesta* (Lepidoptera: Tortricidae) to plant derived volatiles. *Bulletin of Entomological Research* **93**(4): 335-342.

Nomura K, Melotto M, He S-Y. 2005. Suppression of host defense in compatible plant–*Pseudomonas syringae* interactions. *Current Opinion in Plant Biology* **8**(4): 361-368.

Nürnberger T, Lipka V. 2005. Non-host resistance in plants: new insights into an old phenomenon. *Molecular Plant Pathology* **6**(3): 335-345.

Ossipov V, Klemola T, Ruohomäki K, Salminen J-P. 2014. Effects of three years' increase in density of the geometrid *Epirrita autumnata* on the change in metabolome of mountain birch trees (*Betula pubescens* ssp. *czerepanovii*). *Chemoecology* **24**(5): 201-214.

Pare P, Tumlinson J. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiology* **121**: 325 - 331.

Paré PW, Tumlinson JH. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiology* **114**(4): 1161-1167.

Pashalidou FG, Gols R, Berkhout BW, Weldegergis BT, van Loon JJA, Dicke M, Fatouros NE. 2014. To be in time: egg deposition enhances plant-mediated detection of young caterpillars by parasitoids. *Oecologia* **177**(2): 477-486.

Penaflor MF, Erb M, Robert CA, Miranda LA, Werneburg AG, Dossi FC, Turlings TC, Bento JM. 2011. Oviposition by a moth suppresses constitutive and herbivore-induced plant volatiles in maize. *Planta* **234**(1): 207-215.

Penninckx IAMA, Thomma BPHJ, Buchala A, Métraux J-P, Broekaert WF. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *The Plant Cell Online* **10**(12): 2103-2114.

Peñuelas J, Llusià J. 2003. BVOCs: plant defense against climate warming? *Trends in Plant Science* **8**(3): 105-109.

Peñuelas J, Llusià J. 2004. Plant VOC emissions: making use of the unavoidable. *Trends in Ecology & Evolution* **19**(8): 402-404.

Pichersky E, Gershenzon J. 2002. The formation and function of plant volatiles: perfumes for pollinator

attraction and defense. *Current Opinion in Plant Biology* **5**(3): 237-243.

Pichersky E, Noel JP, Dudareva N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* **311**(5762): 808-811.

Piel J, Atzorn R, Gäbler R, Kühnemann F, Boland W. 1997. Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. *FEBS Letters* **416**(2): 143-148.

Pierre P, Jansen J, Hordijk C, van Dam N, Cortesero A-M, Dugravot S. 2011. Differences in volatile profiles of turnip plants subjected to single and dual herbivory above- and belowground. *Journal of Chemical Ecology* **37**(4): 368-377.

Piesik D, Lemńczyk G, Skoczek A, Lamparski R, Bocianowski J, Kotwica K, Delaney KJ. 2011. *Fusarium* infection in maize: volatile induction of infected and neighboring uninfected plants has the potential to attract a pest cereal leaf beetle, *Oulema melanopus*. *Journal of Plant Physiology* **168**(13): 1534-1542.

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

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM. 2009. Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5**(5): 308-316.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM. 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology* **28**(1): 489-521.

Pineda A, Dicke M, Pieterse CMJ, Pozo MJ. 2012. Beneficial microbes in a changing environment: are they always helping plants to deal with insects? *Functional Ecology* **27**: 574–586.

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

Poelman EH, Bruinsma M, Zhu F, Weldegergis BT, Boursault AE, Jongema Y, van Loon JJA, Vet LEM, Harvey JA, Dicke M. 2012. Hyperparasitoids use herbivore-induced plant volatiles to locate their parasitoid host. *PLoS Biology* **10**(11): e1001435.

Poelman EH, Oduor AMO, Broekgaarden C, Hordijk CA, Jansen JJ, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M. 2009. Field parasitism rates of caterpillars on *Brassica oleracea* plants are reliably predicted by differential attraction of *Cotesia* parasitoids. *Functional Ecology* **23**(5): 951-962.

Poelman EH, van Loon JJA, Dicke M. 2008b. Consequences of variation in plant defense for biodiversity at higher trophic levels. *Trends in Plant Science* **13**(10): 534-541.

Poelman EH, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M. 2010. Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. *Ecological Entomology* **35**(2): 240-247.

Ponzio C, Cascone P, Cusumano A, Weldegergis BT, Fatouros NE, Guerrieri E, Dicke M, Gols R. 2016. Volatile-mediated foraging behaviour of three parasitoid species under conditions of dual insect herbivore attack. *Animal Behaviour* **111**: 197-206.

Ponzio C, Gols R, Pieterse CMJ, Dicke M. 2013. Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. *Functional Ecology* **27**(3): 587-598.

Ponzio C, Gols R, Weldegergis BT, Dicke M. 2014. Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore. *Plant, Cell & Environment* **37**(8): 1924-1935.

Pope T, Kissen R, Grant M, Pickett J, Rossiter J, Powell G. 2008. Comparative innate responses of the aphid parasitoid *Diaeretiella rapae* to alkenyl glucosinolate derived isothiocyanates, nitriles, and

epithionitriles. *Journal of Chemical Ecology* **34**(10): 1302-1310.

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

Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J. 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Molecular Biology* **42**(1): 93-113.

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

Rasmann S, Turlings T. 2007. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecology Letters* **10**(10): 926 - 936.

Ratzka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J. 2002. Disarming the mustard oil bomb. *Proceedings of the National Academy of Sciences* **99**(17): 11223-11228.

Reddy GVP 2012. Recent trends in the olfactory responses of insect natural enemies to plant volatiles. In: Witzany G, Baluška F eds. *Biocommunication of plants*: Springer Berlin Heidelberg, 281-301.

Robert-Seilaniantz A, Grant M, Jones JD. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology* **49**(1): 317-343.

Rodriguez-Saona C, Chalmers J, Raj S, Thaler J. 2005. Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. *Oecologia* **143**(4): 566 - 577.

Rodriguez-Saona C, Crafts-Brandner SJ, Cañas LA. 2003. Volatile emissions triggered by multiple herbivore damage: beet armyworm and whitefly feeding on cotton plants. *Journal of Chemical Ecology* **29**(11): 2539-2550.

Rostás M, Simon M, Hilker M. 2003. Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. *Basic and Applied Ecology* **4**(1): 43-62.

Rostás M, Ton J, Mauch-Mani B, Turlings T. 2006. Fungal infection reduces herbivore-induced plant volatiles of maize but does not affect naive parasitoids. *Journal of Chemical Ecology* **32**(9): 1897-1909.

Rowen E, Kaplan I. 2016. Eco-evolutionary factors drive induced plant volatiles: a meta-analysis. *New Phytologist* **210**(1): 284-294.

Sabelis M, Van De Baan H. 1983. Location of distant spider mite colonies by phytoseiid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomologia Experimentalis et Applicata* **33**(3): 303-314.

Sarmento RA, Lemos F, Bleeker PM, Schuurink RC, Pallini A, Oliveira MG, Lima ER, Kant M, Sabelis MW, Janssen A. 2011. A herbivore that manipulates plant defence. *Ecology Letters* **14**(3): 229-236.

Scala A, Allmann S, Mirabella R, Haring MA, Schuurink RC. 2013. Green leaf volatiles: a plant's multifunctional weapon against herbivores and pathogens. *International Journal of Molecular Sciences* **14**(9): 17781-17811.

Schoonhoven L, Van Loon J, Dicke M. 2005. *Insect-plant biology*: Oxford University Press, USA.

Schwachtje J, Baldwin IT. 2008. Why does herbivore attack reconfigure primary metabolism? *Plant Physiology* **146**(3): 845-851.

Schwartzberg EG, Boroczky K, Tumlinson JH. 2011. Pea aphids, *Acyrthosiphon pisum*, suppress induced plant volatiles in broad bean, Vicia faba. *Journal Chemical Ecology* **37**(10): 1055-1062.

Shiojiri K, Kishimoto K, Ozawa R, Kugimiya S, Urashimo S, Arimura G, Horiuchi J, Nishioka T, Matsui K, Takabayashi J. 2006. Changing green leaf volatile biosynthesis in plants: an approach for improving plant resistance against both herbivores and pathogens. *Proceedings of the National*

Academy of Sciences **103**(45): 16672-16676.

Shiojiri K, Ozawa R, Kugimiya S, Uefune M, van Wijk M, Sabelis MW, Takabayashi J. 2010. Herbivore-specific, density-dependent induction of plant volatiles: honest or "cry wolf" signals? *PLoS One* **5**(8): e12161.

Shiojiri K, Takabayashi J, Yano S, Takafuji A. 2000. Flight response of parasitoids toward plant-herbivore complexes: a comparative study of two parasitoid-herbivore systems on cabbage plants. *Applied Entomology and Zoology* **35**(1): 87-92.

Shiojiri K, Takabayashi J, Yano S, Takafuji A. 2001. Infochemically mediated tritrophic interaction webs on cabbage plants. *Population Ecology* **43**(1): 23-29.

Shiojiri K, Takabayashi J, Yano S, Takafuji A. 2002. Oviposition preferences of herbivores are affected by tritrophic interaction webs. *Ecology Letters* **5**(2): 186-192.

Shulaev V, Silverman P, Raskin I. 1997. Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* **385**(6618): 718-721.

Singh V, Louis J, Ayre BG, Reese JC, Shah J. 2011. TREHALOSE PHOSPHATE SYNTHASE11-dependent trehalose metabolism promotes *Arabidopsis thaliana* defense against the phloem-feeding insect *Myzus persicae*. *The Plant Journal* **67**(1): 94-104.

Singh V, Shah J. 2012. Tomato responds to green peach aphid infestation with the activation of trehalose metabolism and starch accumulation. *Plant Signaling & Behavior* **7**(6): 605-607.

Smid HM, van Loon JJA, Posthumus MA, Vet LEM. 2002. GC-EAG-analysis of volatiles from Brussels sprouts plants damaged by two species of *Pieris* caterpillars: olfactory receptive range of a specialist and a generalist parasitoid wasp species. *Chemoecology* **12**(4): 169-176.

Sohrabi R, Huh J-H, Badieyan S, Rakotondraibe LH, Kliebenstein DJ, Sobrado P, Tholl D. 2015. In planta variation of volatile biosynthesis: An alternative biosynthetic route to the formation of the pathogen-induced volatile homoterpene DMNT via triterpene degradation in Arabidopsis roots. *The Plant Cell* **27**(3): 874-890.

Soler R, Badenes-Pérez FR, Broekgaarden C, Zheng S-J, David A, Boland W, Dicke M. 2012a. Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. *Functional Ecology* **26**(1): 156-166.

Soler R, Harvey JA, Kamp AFD, Vet LEM, Van der Putten WH, Van Dam NM, Stuefer JF, Gols R, Hordijk CA, Martijn Bezemer T. 2007. Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals. *Oikos* **116**(3): 367-376.

Soler R, Pineda A, Li Y, Ponzio C, van Loon JJA, Weldegergis BT, Dicke M. 2012b. Neonates know better than their mothers when selecting a host plant. *Oikos* **121**(12): 1923-1934.

Spoel SH, Dong X. 2008. Making sense of hormone crosstalk during plant immune responses. *Cell Host & Microbe* **3**(6): 348-351.

Spoel SH, Johnson JS, Dong X. 2007. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences* **104**(47): 18842-18847.

Stam JM, Kroes A, Li Y, Gols R, van Loon JJA, Poelman EH, Dicke M. 2014. Plant interactions with multiple insect herbivores: from community to genes. *Annual Review of Plant Biology* **65**(1): 689-713.

Steinbrenner A, Gómez S, Osorio S, Fernie A, Orians C. 2011. Herbivore-induced changes in tomato (*Solanum lycopersicum*) primary metabolism: A whole plant perspective. *Journal of Chemical Ecology* **37**(12): 1294-1303.

Stout MJ, Fidantsef AL, Duffey SS, Bostock RM. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* **54**(3-4): 115-130.

Stout MJ, Thaler JS, Thomma BPHJ. 2006. Plant-mediated interactions between pathogenic

microorganisms and herbivorous arthropods. *Annual Review of Entomology* **51**(1): 663-689.

Strange RN, Scott PR. 2005. Plant disease: a threat to global food security. *Annual Review of Phytopathology* **43**: 83-116.

Sutter R, Müller C. 2011. Mining for treatment-specific and general changes in target compounds and metabolic fingerprints in response to herbivory and phytohormones in *Plantago lanceolata*. *New Phytologist* **191**(4): 1069-1082.

Tack AJM, Dicke M. 2013. Plant pathogens structure arthropod communities across multiple spatial and temporal scales. *Functional Ecology* **27**(3): 633-645.

Tack AJM, Gripenberg S, Roslin T. 2012. Cross-kingdom interactions matter: fungal-mediated interactions structure an insect community on oak. *Ecology Letters* **15**(2): 177-185.

Takabayashi J, Dicke M, Posthumus MA. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. *Chemoecology* **2**(1): 1-6.

Takabayashi J, Takahashi S, Dicke M, Posthumus MA. 1995. Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *Journal of Chemical Ecology* **21**(3): 273-287.

Tamiru A, Bruce TJ, Woodcock CM, Caulfield JC, Midega CA, Ogol CK, Mayon P, Birkett MA, Pickett JA, Khan ZR. 2011. Maize landraces recruit egg and larval parasitoids in response to egg deposition by a herbivore. *Ecology Letters* **14**(11): 1075-1083.

Tasin M, Knudsen GK, Pertot I. 2012. Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. *Animal Behaviour* **83**(2): 555-562.

Textor S, Gershenzon J. 2009. Herbivore induction of the glucosinolate–myrosinase defense system: major trends, biochemical bases and ecological significance. *Phytochemistry Reviews* **8**(1): 149-170.

Thaler JS, Fidantsef AL, Bostock RM. 2002. Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. *Journal of Chemical Ecology* **28**(6): 1131-1159.

Thaler JS, Humphrey PT, Whiteman NK. 2012. Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science* **17**(5): 260-270.

Thaler JS, Owen B, Higgins VJ. 2004. The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. *Plant Physiology* **135**(1): 530-538.

Titayavan M, Altieri MA. 1990. Synomone-mediated interactions between the parasitoid *Diaeretiella rapae* and *Brevicoryne brassicae* under field conditions. *Entomophaga* **35**(4): 499-507.

Ton J, Van Pelt JA, Van Loon LC, Pieterse CMJ. 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. *Molecular Plant-Microbe Interactions* **15**(1): 27-34.

Tooker JF, Rohr JR, Abrahamson WG, De Moraes CM. 2008. Gall insects can avoid and alter indirect plant defenses. *New Phytologist* **178**(3): 657-671.

Truong D-H, Delory B, Vanderplanck M, Brostaux Y, Vandereycken A, Heuskin S, Delaplace P, Francis F, Lognay G. 2014. Temperature regimes and aphid density interactions differentially influence VOC emissions in Arabidopsis. *Arthropod-Plant Interactions* **8**(4): 317-327.

Trygg J, Wold S. 2002. Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics* **16**(3): 119-128.

Tumlinson JH, Engelberth J 2008. Fatty acid-derived signals that induce or regulate plant defenses against herbivory. In: Schaller A ed. *Induced Plant Resistance to Herbivory*: Springer Netherlands, 389-407.

Turlings T, Tumlinson J, Lewis W. 1990. Exploitation of herbivore-induced plant odors by host-seeking

parasitic wasps. *Science* **250**(4985): 1251 - 1253.

Turlings T, Wackers F 2004. Recruitment of predators and parasitoids by herbivore-damaged plants. In: Cardé RT, Millar JG eds. *Advances in Insect Chemical Ecology*, 21 - 75.

Turlings TC, Fritzsche ME 1999. Attraction of parasitic wasps by caterpillar-damaged plants. In: Goode DJCaJ ed. *Insect-Plant Interactions and Induced Plant Defence (Novartis Foundation Symposium)*. Chicester: John Wiley & Sons, 21-32.

Turlings TC, Loughrin JH, McCall PJ, Röse US, Lewis WJ, Tumlinson JH. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proceedings of the National Academy of Sciences* **92**(10): 4169-4174.

Turlings TCJ, Lengwiler UB, Bernasconi ML, Wechsler D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* **207**(1): 146-152.

Turlings TCJ, Tumlinson JH, Lewis WJ. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* **250**(4985): 1251-1253.

Unsicker SB, Kunert G, Gershenzon J. 2009. Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. *Current Opinion in Plant Biology* **12**(4): 479-485.

van Alphen JJM, Bernstein C, Driessen G. 2003. Information acquisition and time allocation in insect parasitoids. *Trends in Ecology & Evolution* **18**(2): 81-87.

van Molken T, de Caluwe H, Hordijk CA, Leon-Reyes A, Snoeren TA, van Dam NM, Stuefer JF. 2012. Virus infection decreases the attractiveness of white clover plants for a non-vectoring herbivore. *Oecologia* **170**(2): 433-444.

van Mölken T, Kuzina V, Munk K, Olsen C, Sundelin T, van Dam N, Hauser T. 2014. Consequences of combined herbivore feeding and pathogen infection for fitness of *Barbarea vulgaris* plants. *Oecologia* **175**(2): 589-600.

van Poecke RMP, Dicke M. 2004. Indirect defence of plants against herbivores: using *Arabidopsis thaliana* as a model plant. *Plant Biology* **6**(4): 387-401.

van Tol RWHM, Visser JH. 2002. Olfactory antennal responses of the vine weevil *Otiorhynchus sulcatus* to plant volatiles. *Entomologia Experimentalis et Applicata* **102**(1): 49-64.

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

Verhage A, van Wees SCM, Pieterse CMJ. 2010. Plant immunity: it's the hormones talking, but what do they say? *Plant Physiology* **154**(2): 536-540.

Verhage A, Vlaardingerbroek I, Raaymakers C, Van Dam NM, Dicke M, Van Wees SC, Pieterse CM. 2011. Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory. *Frontiers in Plant Science* **2**: 47.

Vet L 1996. Parasitoid foraging: the importance of variation in individual behaviour for population dynamics. In: Floyd R, Sheppard A eds. *Frontiers of Population Ecology*. Melbourne: CSIRO Publishing, 245-256.

Vet L, Dicke M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annual Review of Entomology* **37**(1): 141-172.

Visser JH, Avé DA. 1978. General green leaf volatiles in the olfactory orientiation of the colorado potato beetle, *Leptinotarsa decemlineata*. *Entomologia Experimentalis et Applicata* **24**(3): 738-749.

Viswanathan DV, Lifchits OA, Thaler JS. 2007. Consequences of sequential attack for resistance to herbivores when plants have specific induced responses. *Oikos* **116**(8): 1389-1399.

von Dahl C, Baldwin I. 2007. Deciphering the role of ethylene in plant-herbivore interactions. *Journal of Plant Growth Regulation* **26**(2): 201-209.

Vos M, Berrocal SM, Karamaouna F, Hemerik L, Vet LEM. 2001. Plant-mediated indirect effects and the persistence of parasitoid–herbivore communities. *Ecology Letters* **4**(1): 38-45.

Walling LL. 2000. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation* **19**(2): 195 - 216.

Walters DR. 2011. *Plant defense : warding off attack by pathogens, pests and vertebrate herbivores*. Oxford: Wiley-Blackwell.

Widarto H, Van Der Meijden E, Lefeber AM, Erkelens C, Kim H, Choi Y, Verpoorte R. 2006. Metabolomic differentiation of *Brassica rapa* following herbivory by different insect instars using two-dimensional nuclear magnetic resonance spectroscopy. *Journal of Chemical Ecology* **32**(11): 2417-2428.

Winde I, Wittstock U. 2011. Insect herbivore counteradaptations to the plant glucosinolate–myrosinase system. *Phytochemistry* **72**(13): 1566-1575.

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenzon J, Vogel H. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences of the United States of America* **101**(14): 4859-4864.

Wyatt TD. 2003. *Pheromones and animal behaviour: communication by smell and taste*. Cambridge, UK: Cambridge University Press.

Yao I, Akimoto S-i. 2001. Ant attendance changes the sugar composition of the honeydew of the drepanosiphid aphid *Tuberculatus quercicola*. *Oecologia* **128**(1): 36-43.

Yoneya K, Miki T. 2015. Co-evolution of foraging behaviour in herbivores and their natural enemies predicts multifunctionality of herbivore-induced plant volatiles. *Functional Ecology* **29**(4): 451-461.

Zarate SI, Kempema LA, Walling LL. 2007. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Physiology* **143**(2): 866-875.

Zhang P-J, Broekgaarden C, Zheng S-J, Snoeren TAL, van Loon JJA, Gols R, Dicke M. 2013. Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. *New Phytologist* **197**(4): 1291-1299.

Zhang P-J, Zheng S-J, van Loon JJA, Boland W, David A, Mumm R, Dicke M. 2009. Whiteflies interfere with indirect plant defense against spider mites in Lima bean. *Proceedings of the National Academy of Sciences* **106**(50): 21202-21207.

Summary

While plants appear to lead a simple and unexciting existence, they are actually members of complex and dynamic environments, and are under constant threat from a wide range of attackers, which includes organisms such as insect herbivores or plant pathogens. Plants are far from being passive against this onslaught, and possess a sophisticated arsenal of defense mechanisms, which can be broadly categorized into constitutive and induced defenses. Constitutive defenses represent a first line of defense; they are constantly expressed in plants, and consist of e.g. thorns, trichomes or toxins. Induced defenses are activated at the onset of attack, and can be further separated into two categories: induced direct defenses which negatively affect the attackers and include e.g. the production of toxic chemical compounds, and induced indirect defenses which promote the effectiveness of natural enemies of the herbivores such as predators or parasitoid wasps, e.g. via the production of herbivore-induced plant volatiles (HIPV).

However, while the production and use of HIPV by foraging natural enemies of herbivores has been well studied over the years, it has been limited to studies on one herbivore and parasitoid combination. In natural situations it is far more common for a plant to be challenged by multiple attackers, either simultaneously or sequentially. The presence of a second attacker has strong potential to modify plant responses to the first attack. Yet while there is information on how multiple attack affects insect fitness and plant responses at the molecular level, a lot less is known about how plant chemistry and the foraging behavior of natural enemies of herbivores are affected, especially when one of the secondary attackers is a plant pathogen.

The aim of this thesis was to explore how dual attack modifies plant chemistry and how these changes in the plant then affect the behavior of foraging parasitoid wasps via the induced volatile blend, with a strong focus on the effects of non-host herbivore density and plant pathogen challenge. The main focus of the study was on the tritrophic system consisting of wild black mustard plants (*Brassica nigra*), the large cabbage white butterfly, *Pieris brassicae*, and its larval parasitoid, *Cotesia glomerata*. This study system is naturally occurring in the Netherlands, allowing for the study of ecologically relevant interactions between plant and insects.

To gain deeper insight into this topic, the project started with a review of the current literature dealing with the effects of single or combined insect herbivore or plant pathogen challenge on the induction and ecological role of plant volatiles. This review highlighted the fact that knowledge of volatile induction under single attack cannot be extrapolated to dual attack scenarios, and though studies including plant pathogens were rare, they showed a strong potential for pathogens to modify the volatile cues used by natural

enemies of insect herbivores. I concluded that further studies incorporating plant–insect and plant–pathogen interactions at different levels of biological organization are needed to provide insight in how plants integrate cues from different groups of attacking organisms into an adaptive defense response.

The experimental work started in Chapter 3 by comparing the effects of three different types of non-host attackers on the focal tritrophic system in order to discover if the effects of dual attack are attacker specific. These non-host attackers were either eggs of *P. brassicae*, phloem-feeding cabbage aphids (*Brevicoryne brassicae*) or a plant pathogen specialized on brassicaceous plant species (*Xanthomonas campestris* pv. *campestris*). The induced volatile blends of plants subjected to single and dual herbivory were investigated, and *C. glomerata* parasitoid behavioral responses to various attack scenarios were evaluated. It was discovered that despite the highly contrasting nature of the attackers, the volatile blends exploited by *C. glomerata* wasps were more robust to interference by other challengers than was often assumed, and the wasps could successfully locate their hosts on dually attacked plants. However, it was noted that non-host herbivore density and pathogen challenge could potentially be important factors in modifying plant volatiles and parasitoid behavior: the preference for healthy plants over aphid-infested plants depended on aphid density, and plants challenged with only the pathogen were highly attractive to the wasps. These two aspects were further investigated in the following chapters.

In Chapter 4, the effect of aphid (*B. brassicae*) infestation on the foraging behavior of three parasitoid species was evaluated, while considering the role of aphid density. Aphids were the host for one parasitoid species (*Diaeretiella rapae*), and the non-host for the two other, which were the larval parasitoid *C. glomerata*, and the egg parasitoid *Trichogramma brassicae*. Remarkably, dual infestation with aphids affected the foraging behavior of all three parasitoid species though not in the same manner for each species. While *T. brassicae* preference was generally negatively affected by aphid presence regardless of density, *C. glomerata* and *D. rapae* behavior was negatively affected by increasing density. In the case of *D. rapae*, where three densities were tested, the increasing density also had a negative and linear effect on foraging behavior. In contrast, analysis of the induced volatiles showed that while aphid-density effects were present, the plant response to density was non-linear, creating a strong dichotomy between plant volatile and parasitoid response. This dose-response approach revealed that correlations between volatile and behavioral responses based on commonly used simple experiments should be interpreted cautiously. A role for spatial distribution of the attackers was also found, as herbivores were located on the same leaf rather than adjacent leaves (Chapter 3) which led to clear

density-dependent effects on *C. glomerata* behavior.

In Chapter 5 the effect of plant pathogen challenge on plant volatiles and the foraging behavior of *C. glomerata* was investigated more in-depth. Since it is known that pathogens can differentially affect plant responses at the transcriptomic and phytohormonal level depending on whether the strain used is virulent or avirulent on the plants (i.e. causes disease, or not), the role of virulence was considered in this chapter by including the virulent strain *X. campestris* pv. *campestris* and the avirulent strain *X. campestris* pv. *incanae*. The results showed that a plant-pathogen interaction does not need to be compatible (i.e. successful infection) for there to be strong effects on plant volatile emissions, and their subsequent exploitation by carnivorous insects. The two tested pathogen strains induced the production of volatile blends that were qualitatively different from each other and from the caterpillar-induced volatile blend, with the virulent strain also inducing several compounds in a similar manner to herbivory. Disease severity also had a strong impact on the induced volatile blends, with volatiles from plants having mild or moderate symptoms clearly separating from those emitted by strongly symptomatic plants. *C glomerata* parasitoid wasps were highly attracted to volatiles from all pathogen challenged treatments, even when there were no host caterpillars on the plant. The effect of pathogen infection, including avirulent strains, can, and should, be considered as they can have equally strong consequences as herbivores on plant volatile induction and the volatile-mediated foraging of parasitoid wasps.

As strong aphid density-dependent effects were found on the induced volatile blends and foraging behavior of parasitoid wasps during single or dual attack, Chapter 6 further examined if these effects could be revealed at the level of leaf chemistry. This chapter investigated metabolome-wide effects of single or dual herbivory on *B. nigra* plants by *B. brassicae* aphids and *P. brassicae* caterpillars, while also considering the effect of aphid density. A comprehensive analysis of the metabolome was done in order to reveal induction patterns in both primary and secondary metabolism, which are typically associated with growth and defense, respectively. The multivariate data analysis allowed the identification of general effects of herbivore identity and density on the various pools of metabolites, and revealed treatment-specific metabolomic profiles. The effects were largely driven by alterations in the glucosinolate and sugar pools, and changes in antioxidant-related metabolites showed that aphid infestation leads to a strong stress response in the plants. This study shows that analyzing the plant metabolome as a single entity rather than as individual metabolites provides new insights into the subcellular processes underlying plant defense against multiple herbivore attackers.

The data presented in this thesis contribute to our understanding of how dual attack affects the chemistry of *B. nigra* plants, and modifies plant interactions with the natural enemies of attacking herbivores. Taken together, the studies reveal that 1) plant pathogen challenge can have as much of an impact as insect herbivory on induced plant volatiles and their use by foraging parasitoids, and 2) focusing only on general species-specific effects of dual attack is too simplistic of an approach, as modifying factors within a dual attack scenario, such a herbivore density or pathogen virulence, can lead to even greater changes in plant chemistry and parasitoid behavior, and thus should not be neglected. The research contained in this thesis demonstrates that it is imperative to work towards greater integration of research on plant-insect and plant-pathogen interactions, as plant defenses to insect and microbial attackers are strongly intertwined. This multidisciplinary approach is important to further our knowledge of the modulation of induced plant defenses in response to increasingly complex attack scenarios. Though we are still a long way from having a thorough understanding of the intricate processes underlying plant-mediated interactions with carnivorous insects during dual attack, including more complexity in future research rather than less, as done in this thesis, will allow scientists to gain a deeper insight on how plants modulate their defense responses against dual or multiple attackers.

ACKNOWLEDGEMENTS

First of all, wow, what a ride the past 4.5 years have been! Embarking on the PhD journey has been one of the hardest, yet most rewarding and the best period of my life. I'm happy and proud to have finished it, yet sad that that wonderful life chapter is coming to a close. I wasn't alone in this journey, which is what made it so fantastic and fun, and also made so much of this thesis possible.

First of all, thank you to my supervisors, Marcel Dicke and Rieta Gols for always supporting me, giving me freedom in my research and for your help when it was needed. Marcel thank you for giving me the chance to do a PhD with you, and for being available no matter how busy you were, even if it meant sending manuscript revisions very late in the evening, during weekends, or while you were traveling. I have a deep admiration for your intense level of commitment and dedication to your group, and it is part of what makes you a great scientist and great leader. Rieta, I could not have asked for a better supervisor! I never lost sight of how incredibly lucky I was to have a daily supervisor that I didn't have to 'share' too much. ☺ You've taught me so much over the years, and made me into not just a better scientist, but also a better person. Thank you for tolerating me showing up in your office with a 'short question' that often turned into a long discussion, and not always all about work! Over the years you've become more than just a supervisor to me, you are also a friend.

Of course I need to give a big, heart-felt thank you to my A-BIO-VOC team members all over Europe. It was a pleasure to work with you and I benefitted from your knowledge and experience across a large range of topics. Christelle, Eliezer and Stefano, it was great to work together as "team Brassica nigra" and meet up at conferences or at EuroVOL meetings (and our mini vacations together!), and thanks to you I learned a lot about transcriptomics and metabolomics, which were two topics which I knew nothing about before starting the PhD journey. Many thanks as well to Antonio, Pasquale, Emilio, and Benedicte, either for carrying out experiments with me, or for your valuable input and comments on manuscripts. Without you several of the chapters in this thesis would not have been completed! Even with these fantastic project partners, most of the work in this thesis wouldn't have happened without the people behind the scenes, who do their very best to produce the plants and many insects needed in the experiments. Thank you to the insect rearing team, and to the people at Unifarm!

The wonderful, cozy, friendly and family-like atmosphere at Ento made my time in the group all the more enjoyable. Dani and Foteini- we have been together almost from the beginning

of our time in Wageningen, celebrating together when each obtained a PhD position, and celebrating again as you both finished and defended, and now it is my turn at last! Thank you for your friendship, the prosecco, the chocolate and all the fun over the years! Enric, Alex, Dani, Foteini, Cindy, Jeltje, Anneke, Jitte, Marjolein, Emma, Lucille, Jenny, Keiko, Ana, and Niels, and everyone else in the lab, thank you for the wonderful working environment over the last 4+ years, and for making coffee breaks and lunch breaks as fun and enjoyable as they were. Thank you to my fellow PhD students for making the PhD trip to Switzerland as fantastic as it was. Thank you to Anneke and Jeltje, it was a pleasure to organize the trip with you, I couldn't have asked for better partners to do this with!

Since sports and friendships were also important for my mental wellbeing these 4 years, I have a few more people to thank. To Tijmen, thank you for circuit training class! As the stress increased in the last year of PhD, lifting weights became a major source of stress relief, even if it meant sacrificing my lunch break twice a week. Thank you all to the members of the Schermutselaers, past and present, it was great to fence with you for so many years, though, alas, I stopped going to trainings in the later part of the PhD. Though I do have to say that attacking you all with a foil/épée/saber is an oddly pleasurable experience after a stressful day/week at work! Many excuses for the bruises, Jan-Willem. And finally, thank you to Marieke and Ernest, for being my fencing buddies, my Vlaam buddies, and the first friends I made when arriving in Wageningen over 8 years ago. You showed me that as a PhD student it was possible to strike that perfect balance between work life and social life, and be good at both.☺ After you left the Netherlands, the frequent trips to Scotland to see you were always welcome breaks even (and especially) when I had a lot of work, and even if these breaks included hiking in truly atrocious conditions at times. A few wee drams and puppy kisses would fix everything! I hope that no matter where the future may take us, we'll always manage to keep this great friendship going.

Last but not least, thank you to my family and my 'other half'. Pierre, I'm so grateful to have you by my side in life, and I know I caused you a lot of worry in my moments of thesis-induced panic and stress, and in my anxiety over what my life and career will be like after the PhD. Thank you for always listening, encouraging me, and helping me stay calm even when things seemed completely impossible. Your pragmatic and down-to-earth perspective on things always helped me through rough moments and it's one of your endearing qualities, that also perfectly balances out my emotional-worrywart side. You are not one to get bogged down in the unimportant stuff, and by trying to take a page out of your book, I now understand how you can so often be stress-free.

To my sister and mother, I know it's not easy to have lived far from each other for so many

years now and I'm sorry that the phone calls and emails were less frequent in the last frantic stretch of writing the thesis. Dear mom, I'm so grateful for all the sacrifices you made since we left the USA over 15 years ago, to give us a chance at the best education possible despite the incredibly challenging circumstances. Without it this book may have never existed. Thank you, and I love you.

ABOUT THE AUTHOR

Camille Ashley Marie Ponzio was born on January 15th 1985 in New Haven, Connecticut, USA. In 1999, she moved to her family's home town, in Lyon, France, where she completed her high school education. Passionate about plants since she was a child, she then completed 2 year applied bachelor program in Horticulture in the neighboring city of Chambéry. In 2006 she started the European Engineer Degree program, which was a dual degree Bachelor program in plant production, biotechnology and agribusiness, between CAH Dronten (now CAH Vilentum University of Applied Sciences) in the Netherlands and the Groupe ESA in Angers, France. It was during these 2 years that she discovered Wageningen University, and her growing interest in plant breeding and phytopathology led her to start the Master of Plant Science program there in 2008. During the MSc she developed a strong interest in plant-insect interactions, and completed a first thesis on plant-mediated inter-guild interactions at the laboratory of Entomology at Wageningen University, followed by a field-based minor thesis on the effects of plant genotype on herbivore communities at the Department of Entomology of Cornell University, USA, under the supervision of Dr. Jennifer Thaler. After obtaining her MSc degree, she then worked as a research assistant, back at the laboratory of Entomology, studying the effect of insecticide-treated bed nets on malaria mosquitoes. In August 2011 she started working towards her PhD, still at the laboratory of Entomology of Wageningen University, under the supervision of Prof. Dr. Marcel Dicke and Dr. Rieta Gols. The PhD project was part of the EU-wide project A-BIO-VOC, part of EuroVOL, which aimed to unravel the induction of plant volatile emissions by biotic and abiotic stresses and the consequences for community ecology.

PUBLICATION LIST

Ponzio C, Papazian S, Albrectsen B, Dicke M, Gols R. **2016.** Dual herbivore attack and herbivore density affect metabolic profiles of *Brassica nigra* leaves. (in preparation)

Ponzio C, Weldegergis BT, Dicke M, Gols R. **2016**. Compatible and incompatible pathogen-plant interactions differentially affect plant volatile emissions and the attraction of parasitoid wasps. Functional Ecology (in press)

Ponzio C, Cascone P, Cusumano A, Weldegergis BT, Fatouros NE, Guerrieri E, Dicke M, Gols R. **2016**. Volatile-mediated foraging behaviour of three parasitoid species under conditions of dual insect herbivore attack. Animal Behaviour 111: 197-206.

Ponzio C, Gols R, Weldegergis BT, Dicke M. **2014**. Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore. Plant, Cell & Environment 37(8): 1924-1935.

Spitzen J, Ponzio C, Koenraadt CJM, Pates Jamet HV, Takken W. **2014**. Absence of close-range excitorepellent effects in malaria mosquitoes exposed to deltamethrin-treated bed nets. The American Journal of Tropical Medicine and Hygiene 90(6): 1124-1132.

Ponzio C, Gols R, Pieterse CMJ, Dicke M. **2013**. Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. Functional Ecology 27(3): 587-598.

Soler R, Pineda A, Li Y, Ponzio C, van Loon JJA, Weldegergis BT, Dicke M. **2012**. Neonates know better than their mothers when selecting a host plant. Oikos 121(12): 1923-1934.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Camille Ponzio
Date: 21 June 2016
Group: Laboratory of Entomology
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Exploring plant volatiles in a multiple attack scenario	Mar 14, 2012
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens, Functional Ecology 2013	2012-2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day 2012, University of Amsterdam	Nov 30, 2012
EPS PhD student day 2013, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
EPS Theme 2 day: Interactions between plants and biotic agents, Wageningen	Feb 10, 2012
EPS Theme 2 day: Interactions between plants and biotic agents, Utrecht	Jan 24, 2013
EPS Theme 2 day: Interactions between plants and biotic agents, Amsterdam	Feb 25, 2014
EPS Theme 2 day: Interactions between plants and biotic agents, Utrecht	Feb 20, 2015
► **Lunteren days and other National Platforms**	
Annual Meeting of the Netherlands Entomological Society	Dec 16, 2011
Annual Meeting of the Netherlands Entomological Society	Dec 14, 2012
Netherlands Annual Ecology Meeting, Lunteren	Feb 07-08, 2012
Netherlands Annual Ecology Meeting , Lunteren	Feb 05-06, 2013
Annual Meeting of the Netherlands Entomological Society	Dec 13, 2013
Netherlands Annual Ecology Meeting, Lunteren	Feb 11-12, 2014
Annual Meeting of the Netherlands Entomological Society	Dec 19, 2014
► **Seminars (series), workshops and symposia**	
EPS Expectations Career day, Wageningen	Nov 18, 2011
6th Workshop Plant Insect Interactions - Amsterdam	Nov 22, 2011

7th Workshop Plant Insect Interactions - Leiden	Nov 28, 2012
EPS Expectations Career day, Wageningen	Feb 01, 2013
8th Workshop Plant Insect Interactions -Wageningen	Sep 24, 2013
Wageningen Evolution and Ecology Seminars (WEES)	
Michael Strand - Viruses as beneficial symbionts of insects	Apr 26, 2012
Patrizia d'Ettorre - Recognition of social indentity in ants: pheromone and signature mixtures	Sep 20, 2012
Nicole van Dam - Multiple-stress management: what can we learn from plants?	Dec 20, 2012
Jacintha Ellers- A novel theory of reductive evolution: How ecological interactions can drive compensated loss of traits	Oct 17, 2013
Joy Bergelson - Maintaining an encient balanced polymorphism for resistance amidst diffuse interactions	Sep 26, 2014
Koos Biesmeijer (Naturalis) - On bees, pollination and food security	Dec 18, 2014
Invited seminars Entomology	
Ayko Tack - The importance of pathogens in structuring arthropod communities across multiple time scales	Feb 29, 2012
Evolution of extremely female biased sex ratio in a parsitoid wasp	Mar 03, 2012
Suresh Raina- Linking forest biodiversity to sustainable livlihoods; can we conserve forest habitats using commercially important insects?	Apr 16, 2012
Maaike Bruinsma- Assessing the effects of genetically modified crops on the insect community using potato as a case study	Apr 24, 2012
Madelaine Beekman - Parasitism,cloning and other strange things in the cape honey bee	Sep 04, 2012
Other seminars	
Evolution of temperature response: From single species to community dynamics	Jan 31, 2012
Bart Pannebajjer- Sex ratio adjustment in insect parasitoids; a genomic perspective	Feb 28, 2012
Flying seminar - Arabidopsis as a model system for the study of evolutionnary questions	Feb 27, 2013
PSG seminar- Metabolomics	Oct 08, 2013
Pieter Zuidema- Editor's perspective of the review process	May 27, 2014
Flying seminar - Noah Whiteman - Evolution of herbivory from within a microbe feeding lineage	Jun 17, 2014
Florian Schiestl - Evolution of floral signals in plants: mechanisms and consequences	Mar 12, 2015
► **Seminar plus**	
WEES seminar and master class with Jacintha Ellers	Oct 17, 2013
► **International symposia and congresses**	
14th Symposium on Insect-Plant Interactions, Wageningen	Aug 13-18, 2011
EuroVOL conference (Florence, Italy)	Apr 03-05, 2013
Gordon conference Plant Volatiles (California, USA)	Jan 25-31, 2014
15th Symposium on Insect-Plant Interactions, (Neuchatel, Switzerland)	Aug 17-22, 2014
► **Presentations**	
A-BIO-VOC project meeting (Talk)	Nov 01-02, 2012
8th Workshop Plant Insect Interactions (Talk)	Sep 24, 2013

	Gordon Conference on Plant Volatiles, California USA (Poster)	Jan 25, 2014
	EPS Theme 2 Symposium & Willie Commelin Scholten day (Talk)	feb 25, 2014
	Final EuroVOL project meeting (after SIP15), Neuchatel CH (Talk)	Aug 22, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
►	**Excursions**	
	PhD trip to Switzerland	Oct 28-Nov 01, 2013
	Subtotal Scientific Exposure	*19.8 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	EUROVOL Summer school on Plant Volatiles, Les Diablerets (Switzerland)	Sep 09-12, 2013
	Introduction to R for statistical analysis	May 19-20, 2014
	Generalized linear models	Jun 16-17, 2014
►	**Journal club**	
	PhD lunch meetings (bi-weekly)	2011-2015
	Insect-plant interaction lunch meetings (bi-weekly)	2011-2015
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.2 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Project and Time Management	Nov-Dec 2012
	Voice Matters - Voice and Presentation Skills Training	Nov 13 & 27, 2012
	Writing Grant Proposals	Sep-Nov 2014
	Last Stretch of the PhD Programme	May 22, 2015
	PhD workshop carousel	Apr 17, 015
	Career Assessment	Sep 16, 2015
	Adobe InDesign Essential Training	Sept 29-30 2015
►	**Organisation of PhD students day, course or conference**	
	PhD scientific excusion to Switzerland	Oct 2013
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*5.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**39.4**

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.*

The research described in this thesis, as part of the European Science Foundation EUROCORES program EuroVOL, was financially supported by a grant from the Earth and Life Sciences Council of the the Netherlands Organization for Scientific Research (NWO-ALW).

Cover & layout design by the author

Printed by GVO drukkers & vormgevers, Ede, The Netherlands