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Infochemical use in *Brassica*–insect interactions

A phenotypic manipulation approach to induced plant defences

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Infochemical use in *Brassica*-insect interactions. A phenotypic manipulation approach to induced plant defences

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ABSTRACT

Plants have developed a range of strategies to defend themselves against herbivore attack. Defences can be constitutive, i.e. always present independent of attack, or induced, i.e. only elicited when the plant is under attack. In this thesis, I focused on induced chemical defence responses of plants and the response of associated insects to these phenotypic changes in plants. Herbivore attack is known to induce chemical defences in Brassicaceous plants. Using several elicitors and inhibitors of different steps of the signalling pathways underlying herbivore-induced plant responses, I studied how induced infochemicals affect interactions with associated insects.

Jasmonic acid (JA) is a key plant hormone in the octadecanoid pathway known to be involved in herbivore-induced plant defences. Application of JA can induce plant responses that are similar, although not identical, to herbivore feeding. Two specialist herbivores of Brassicaceous plants, the butterflies *Pieris rapae* and *P. brassicae*, preferred to oviposit on non-induced plants over JA-induced plants. Development of *P. rapae* caterpillars was shown to be reduced, suggesting that oviposition avoidance on JA-induced plants is adaptive. The levels of glucosinolates, secondary metabolites of Brassicaceous plants that are used by *Pieris* butterflies as oviposition stimulants, could not explain the observed oviposition preference of the butterflies.

JA-induced changes in the plants also affected members of the third trophic level. Volatile emission of JA-induced plants attracted parasitoid wasps to the plants. Parasitoid attraction to JA-induced plants was shown to depend on dose and induction time. However, using JA to induce phenotypic changes had effects different from those induced by herbivores, both chemically and ecologically. Volatile emission of JA-induced and herbivore-induced plants differed; whereas JA-induced plants emitted larger amounts of volatiles, the parasitoids preferred herbivore-induced plants over JA-treated ones.

Early events in plant defence responses, involved in attacker recognition, are damage-induced modulations of ion channel activities resulting in ion imbalances. The fungal elicitor alamethicin, an ion channel-forming peptide mixture, was used to mimic early steps in defence responses. Alamethicin treatment increased attractiveness of plants to parasitoid wasps. Although volatile emission of alamethicin-treated plants was much lower, they were equally attractive as JA-treated plants. This indicates that quality rather than quantity of induced plant volatile blends is important to parasitoids.

Besides chemical elicitation of herbivore-induced responses, which is a widely applied approach, plant defence responses can also be chemically inhibited. This provides the opportunity to inhibit the rate of specific enzymatic steps in a signal-transduction pathway. Furthermore, visual cues associated with feeding damage can be present (and similar) in control- and inhibitor-treated plants. Phenidone is a compound that inhibits lipoxygenase, an enzyme catalyzing an early step in the octadecanoid pathway. Parasitoid attraction was reduced when the plants were treated with phenidone before infestation.

Also herbivore oviposition preference was shown to be affected by inhibition of this signalling pathway. Herbivores can differ in their oviposition preferences. I studied two specialist herbivores with different oviposition preferences: *Pieris brassicae* avoids oviposition on herbivore-induced plants, whereas *Plutella xylostella* prefers to oviposit on *Pieris*-infested plants. I showed that these preferences have a chemical basis and are dependent on octadecanoid

signalling, since treatment with the lipoxygenase inhibitor phenidone eliminated herbivore-induced oviposition avoidance or preference.

Thus far, most of the studies on induced plant defences have been done with vegetative plants. However, since reproduction and defence are both processes that require energy and nutrients, this could result in a trade-off. Herbivore feeding on leaves, flowers or roots is known to affect pollinator visitation, but the mechanisms mediating this change have not been addressed. Effects of induction with JA on nectar secretion and pollinator visitation to flowers were investigated. JA-induced plants secreted less nectar, but the sugar concentrations did not change. Also visitation of honeybees and syrphid flies did not change upon JA induction.

These results show the complexity of induced plant defence responses and the variety of behavioural responses of insects on different trophic levels. Combining the phenotypic manipulation approach to induced plant defences, as used in this thesis, with molecular genetic techniques and building on recent developments in plant biochemistry provides a promising way forward towards enhanced understanding of the intricate interactions between plants and insects.

CONTENTS

1
General introduction
Maaike Bruinsma 13

2
Herbivore-induced indirect defence: from induction mechanisms to community ecology
Maaike Bruinsma and Marcel Dicke (2008) 21
Book chapter in 'Induced plant resistance to herbivory', A. Schaller (ed.)

3
Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores
Maaike Bruinsma, Nicole M. Van Dam, Joop J.A. Van Loon & Marcel Dicke (2007) 45
Journal of Chemical Ecology 33:655-668

4
Jasmonic acid-induced changes in *Brassica oleracea* attract parasitoids: effects of time and dose and differences with induction by herbivores
Maaike Bruinsma, Maarten A. Posthumus, R. Mumm, Joop J.A. Van Loon & Marcel Dicke 59

5
Differential effects of jasmonic acid treatment of *Brassica nigra* on the attraction of pollinators, parasitoids and butterflies
Maaike Bruinsma, Harm IJdema, Joop J.A. Van Loon & Marcel Dicke (in press) 77
Entomologia Experimentalis et Applicata

6
Comparing the effects of the fungal elicitor alamethicin and the phytohormone jasmonic acid on volatile emission by *Brassica oleracea* and on attraction of the parasitoid *Cotesia glomerata*
Maaike Bruinsma & Baoping Pang, Roland Mumm, Joop J.A. Van Loon & Marcel Dicke 91

7
Effect of the lipoxygenase-inhibitor phenidone on plant response to herbivore feeding and behavioural responses of a parasitoid and three specialist herbivores
Maaike Bruinsma, Erik H. Poelman, Sarah Van Broekhoven, Maarten A. Posthumus, Martin J. Mueller, Joop J.A. Van Loon & Marcel Dicke 107

8
Summarising discussion: induced plant defence in a community context
Maaike Bruinsma 125

Samenvatting 139
References 145
Dankwoord/Acknowledgements 163
Curriculum vitae 167
Publications 169

Chapter 1

NINA FATOUROS

General introduction

Maaike Bruinsma

Direct and indirect plant defence

During their lifetime most plants have to cope with herbivore attack. The most abundant and diverse group of herbivores attacking plants consist of insects. Almost half of all insect species feed on plants (Schoonhoven et al., 2005). Plants have developed a wide range of physical and chemical mechanisms to defend themselves against herbivore attack, in the form of thorns, surface waxes, trichomes, toxins, extrafloral nectar and shelter for the herbivores' enemies. The defence strategies can be constitutive, meaning that they are always present in the plant independent of herbivore attack; or inducible, meaning that they are only activated when the plant is attacked (Karban and Baldwin, 1997). Both constitutive and induced defence strategies can be divided into direct and indirect defence. Direct defence acts directly on the herbivores, for example by the production of toxins that can deter or kill herbivores. Indirect defence comprises characteristics that promote the effectiveness of natural enemies of the herbivores, for example through the provision of shelter or emission of volatiles that attract predators or parasitoids of the herbivore species feeding on the plant (see also Chapter 2: Bruinsma and Dicke, 2008). For indirect defence the plant strongly depends on the presence of natural enemies of the herbivores in its habitat.

Herbivores: does mother know best?

Insects can use the changes in the plant in response to herbivore damage as sensory cues for the suitability of a plant as a food source. Adult butterflies have to choose a host plant for their offspring to feed and develop on. Since their larvae have only a small action radius, it is important for their survival that they hatch on a plant that can support their feeding and minimises their chances of succumbing to predation and parasitism (Renwick and Chew, 1994). Plants with a lower nutritional value may decrease the growth rate of larvae. The slow-growth-high-mortality hypothesis (Price et al., 1980; Benrey and Denno, 1997) states that chemistry of the plant can influence larval development rate, and thereby the time interval that larvae are vulnerable to parasitism. Oviposition on plants with the fastest development rate for the larvae would benefit the survival of their offspring. Plants that are already under attack by herbivores can contain higher levels of toxins and can be more conspicuous to natural enemies, thereby increasing the risk of predation and parasitism. Therefore, it may be expected that herbivores use induced plant responses to avoid oviposition on attacked plants (but see Shiojiri et al., 2002).

Parasitoids: how to find a host?

The natural enemies of the herbivores, such as parasitoids, need to locate their usually inconspicuous herbivorous hosts. Since their hosts are often small and difficult to detect through host-produced cues, many

parasitoids use other cues. From a distance parasitoids can distinguish between plants with or without hosts feeding on them through volatile cues, and can use these signals to locate their hosts (Turlings et al., 1990; Geervliet et al., 1994). Although plant signals are a less reliable source of information on the location of a host, they are much easier to detect (Vet and Dicke, 1992). Several studies have shown that herbivore-induced plant volatile emission is a more important cue for parasitoids than cues from the host itself, its faeces, or mechanically damaged plants (Steinberg et al., 1993; Geervliet et al., 1994).

Plants: optimising fitness

To defend themselves against herbivore attack, plants use a range of defence strategies. If defence strategies are successful against attackers, why are not all defences constitutive? An important reason is that the production of defence chemicals is a costly process, and energy and nutrients that are used for defence responses cannot be allocated to growth and reproduction (Karban and Baldwin, 1997; Baldwin, 1998). Induced defence can therefore optimise the investment in growth, reproduction and defence against herbivore attack, since it is only induced when necessary. On the other hand, induced defence has the disadvantage that it only becomes effective when the plant is already under attack and this causes a time delay in the defence response.

Another important factor for plant fitness is pollination. Many plant species rely on pollinators for their reproductive success (Myers, 1996; Klein et al., 2007). It is therefore important to optimise flower visitation in terms of number and duration of visitors. Nectar, pollen, flower number, -size and -colour are all important traits that can influence flower visitation and therefore affect pollination rates. However, nectar is not only used by pollinators, like bees and syrphid flies; also herbivores, parasitoids and predators may be attracted to flowers and may feed on their nectar. A trade-off may therefore exist between the attraction of pollinators and defence strategies against herbivores.

Phenotypic manipulation of plant defence

Induced plant responses to herbivory cause phenotypic variation in the plants. This phenotypic plasticity of the plant can be exploited to study the effect of individual plant traits on the insect community associated with the plant (Chapter 2: Bruinsma and Dicke, 2008). Manipulation of the plants' defence response can provide more insight into the mechanisms and ecological consequences of plant defence, by controlled and selective induction of the defence response and excluding the effect of visual or mechanical cues caused by feeding damage. Elicitors and inhibitors of different steps of the signalling pathways involved in defence responses can be used to study their role in the repellence of herbivores

or attraction of their natural enemies. I have experimentally interfered with the jasmonic acid signal-transduction pathway through the application of its key hormone, jasmonic acid, and applied several inhibitors: phenidone, diethyldithiocarbamate and propyl gallate, to study the importance of this pathway in the defence response of Brassicaceous plants. Furthermore, I included one elicitor of both the salicylic acid and octadecanoid pathway, alamethicin, and studied the role of the alamethicin-induced volatiles in the attraction of parasitoids, as well as the interaction with induction of the octadecanoid pathway by jasmonic acid.

Study system

To study the effect of induced defences on the insect community, a system consisting of Brassicaceous plants and several associated insects was used. The tritrophic system consisting of Brussels sprouts plants, cabbage white butterflies and *Cotesia* parasitoids has been extensively used for studies of tritrophic interactions (e.g. Steinberg et al., 1993; Geervliet et al., 1996; Scascighini et al., 2005; Smid et al., 2007). It is a suitable system to study induced plant defence responses because plants and insects are easy to rear; they have no complicated requirements for growth and development. Secondly, there is extensive knowledge on the pests feeding on Brussels sprouts plants and their parasitoids. Thirdly, Brassicaceous plants like Brussels sprouts, *Arabidopsis thaliana* and mustard plants are known to have several defence strategies against herbivorous insects. For example, upon herbivore feeding volatiles are emitted which parasitoids can exploit as cues to find their herbivorous hosts (e.g. Geervliet et al., 1994; Mattiacci et al., 1994; Bukovinszky et al., 2005) and glucosinolates (secondary metabolites of Brassicaceae that can negatively affect herbivores) are often induced upon herbivory (Strauss et al., 2004; Mewis et al., 2005). An additional advantage is that *A. thaliana*, a model species in plant sciences, also for study of defences (Van Poecke et al., 2001; Snoeren et al., 2007), is a Brassicaceous plant. As a result many ecotypes, mutants and transgenic *A. thaliana* plants are available, as well as the entire genome sequence. Studies of related crops species such as Brussels sprouts, can benefit from the knowledge from this

model plant (e.g. Broekgaarden et al., 2007; Zheng et al., 2007). In this thesis most questions were addressed using this system. I used *Brassica nigra,* black mustard, in Chapter 5 (in which I studied pollinators) because, unlike Brussels sprouts, black mustard flowers in the first year.

The cabbage white butterflies, *Pieris rapae* and *P. brassicae* are both specialist herbivores of Brassicaceae. They use glucosinolates as oviposition stimulants (Renwick et al., 1992; Van Loon et al., 1992a; Renwick and Chew, 1994) and the larvae use these secondary metabolites as feeding stimulants (Verschaffelt, 1910; Chew, 1980). *Pieris rapae* is a solitary species which means it lays one egg at a time, while *P. brassicae* is a gregarious species, laying its eggs in clutches.

Cotesia parasitoid wasps are koinobiont larval endoparasitoids, which means they deposit their eggs in larvae of their host and the parasitoid larvae develop in the living host larvae that die after the parasitoids have egressed from the host and pupate. I used *Cotesia rubecula* and *C. glomerata*, both parasitoids of cabbage white caterpillars. They mainly rely on plant volatiles to locate their hosts from a distance (Geervliet et al, 1994). *Cotesia rubecula* is a solitary parasitoid, which means that one parasitoid can develop per caterpillar, while *C. glomerata* is a gregarious parasitoid, in which case many parasitoids can successfully develop within one caterpillar. *Cotesia glomerata* has a higher degree of plasticity in host acceptance behaviour than *C. rubecula*, which is more specialised in its choice of host for oviposition (Brodeur et al., 1996). Both species prefer to parasitise their hosts in the early instars, except for *C. rubecula* that readily accepts third instar *P. rapae* for oviposition (Brodeur et al., 1996).

Other insects that I included in several experiments described in this thesis are the diamondback moth *Plutella xylostella*, a specialist herbivore that feeds on Brassicaceae, and *Diadegma semiclausum*, an Ichneumonid wasp that parasitises *Pl. xylostella* caterpillars. In Chapter 5 I studied flower visitation by pollinators, for which I used honeybees and syrphid flies.

Outline of this thesis

My PhD project is part of an NWO-VICI project which aims to gain understanding of the complex mechanistic processes in plants that are induced by herbivore damage, and to study the consequences for behavioural and community ecology. I approached this from a phenotypic manipulation perspective, while another PhD-project within the same NWO-VICI project took a genotypic approach using different mutant and transgenic *A. thaliana* plants. I have investigated the role of the octadecanoid pathway in plant defence against herbivores by inducing and

inhibiting different steps of this pathway and subsequently studying the effects of the manipulations on interactions of the plants with different community members.

Chapter 2 reviews the literature on herbivore-induced indirect defence, ranging from induction mechanisms to community ecology, and the effects of induced defence on the 1st through the 4th trophic level are discussed. The chapter presents an overview of the current knowledge and identifies the most relevant knowledge gaps to be addressed.

Chapter 3 presents experiments on the role of jasmonic acid (JA) in direct defence of Brussels sprouts plants against specialist herbivores. I have extensively investigated the changes in oviposition preference of a solitary, *Pieris rapae*, and a gregarious, *P. brassicae*, butterfly after JA-treatment of Brussels sprouts plants. I analysed glucosinolate levels in the leaf surface and investigated whether variation in glucosinolates could explain the changes in oviposition preference of the butterflies. Chapter 4 continues to explore the role of JA in induced defence of Brussels sprouts plants, here focussing on indirect defence. I studied the behaviour of three parasitoid wasp species in response to herbivore-infested, JA-treated or control plants, and compared this to the volatile emission by control, JA-treated and herbivore-infested plants. Subsequently, the chapter focused on one of the parasitoid species, *C. glomerata.* Its response to plants induced with different doses of JA was studied, and in a time series it was recorded how long it takes after JA treatment for the plants to become attractive to the parasitoids and how long the JA-treated plant remained attractive. Chapter 5 addresses the question whether the JA-treatment of plants also influences pollinators, besides herbivores and parasitoids. Furthermore, it addresses the question whether the effect of JA-treatment of flowering plants affects the insect community differently than it does in vegetative plants. *Brassica nigra* was chosen as a model plant for this study, and the behaviour of butterflies, parasitoids and pollinators, as well as nectar secretion of the plants were studied in control, JA-treated and herbivore-infested plants.

Chapter 6 addresses the effects of another elicitor, alamethicin (ALA) that not only elicits (part of) the JA-pathway, but also elicits the salicylic acid pathway. Plant volatile emission and the response of the parasitoid *C. glomerata* were investigated after induction of Brussels sprouts plants with ALA, JA or a combination of both. In Chapter 7 I used a different approach to phenotypic manipulation of induced plant defence. Inhibitors were used instead of elicitors to manipulate the induction of the plants. Brussels sprouts plants were treated with three inhibitors that each interfere with a different step in the octadecanoid pathway: phenidone, diethyldithiocarbamate and propyl gallate, and tested their effect on the attractiveness of the plants to the parasitoid *C. glomerata*. Subsequently, for phenidone, the inhibitor with the strongest effect, the response of a second parasitoid, three herbivores, plant volatile emis-

sion as well as levels of a JA-pathway intermediate (downstream of the inhibited step) were recorded.

Finally, Chapter 8 summarises the most important results of the studies in this thesis and discusses them with reference to other results from the studies in the NWO-VICI project and current views in research on multitrophic plant-insect interactions.

Chapter 2

NINA FATOUROS

Herbivore-induced indirect defense: from induction mechanisms to community ecology

Maaike Bruinsma & Marcel Dicke

Book chapter in 'Induced plant resistance to herbivory', A. Schaller (ed.)

Abstract

Herbivory may induce plant defences that promote the activity of natural enemies of the herbivores. This so-called induced indirect defence may involve the production of plant volatiles that attract carnivorous arthropods or extrafloral nectar that is exploited as alternative food by carnivorous arthropods. Induced indirect plant defence is mediated by different signal-transduction pathways, such as the jasmonic acid, the salicylic acid and the ethylene pathways and may involve large-scale transcriptomic re-arrangements. Induced indirect plant defence responses result in an altered phenotype and thus can affect the interactions of the plant with various community members: attackers can be deterred, natural enemies attracted (both above- and belowground), pollinators may change flower visitation, and neighbouring plants can exploit the information from the attacked plants to initiate defence responses as well. We discuss several approaches that are commonly used in molecular, chemical and ecological studies of induced indirect plant defences and identify some remaining knowledge gaps and directions for future research. Integrating a mechanistic approach with a community ecological approach will provide important progress in understanding the selective pressures and dynamics of ecological interactions that are mediated by induced indirect plant defences, as well as the underlying mechanisms.

Introduction

Plants face many challenges during their lifetime. Drought, flooding, high and low temperatures, and attacks by all kinds of organisms, like pathogens, nibbling insects or mammals devouring whole plants. To be able to reproduce in spite of all these difficulties plants have developed defence mechanisms to protect themselves. The defences of plants against herbivorous arthropods can be classified as 'direct defences' that affect the physiology of the attacker or 'indirect defences' that promote the effectiveness of natural enemies of herbivores (Figure 1). Indirect defence comprises (a) the provision of shelter, such as hollow thorns that are used by ants for nesting (b) the production of alternative food, such as extrafloral nectar that is used by carnivorous arthropods such as ants and parasitic wasps or (c) the emission of herbivore-induced plant volatiles that guide carnivorous arthropods such as predators or parasitoids to their herbivorous victims. All of these support the presence and abundance of carnivorous enemies of herbivorous arthropods and consequently the reduction of herbivore presence.

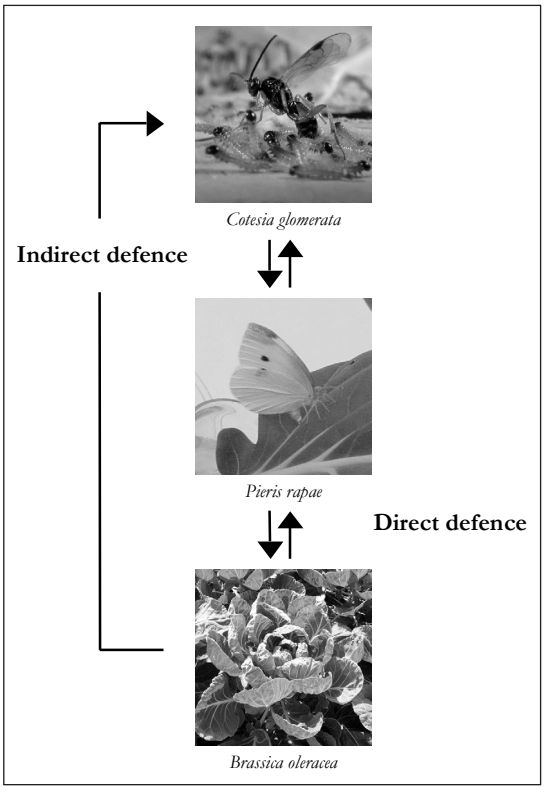


Figure 1. Direct defence of a plant has a direct negative effect of the attacker of the plant. Indirect defence of a plant maintains or attracts carnivores that consume or parasitise the attacker of the plant, thereby exerting a negative effect on the attacker, which contribute to plant defence. Photographs were taken by Hans M. Smid (*C. glomerata*), Nelly Cardinel (*B. oleracea*) and Maaike Bruinsma (*P. rapae*).

Here, we will address indirect defence, especially induced indirect defence. Herbivory can induce the production of extrafloral nectar (Wäckers et al., 2001) or plant volatiles (Van Poecke and Dicke, 2004) and as a result the plant's phenotype changes. The extrafloral nectar can serve as food to various animals in the community and the volatiles can be used by animals to localise the plant. Throughout the plant kingdom many species have been reported to produce herbivore-induced infochemicals that attract carnivorous enemies of the herbivorous arthropods (Dicke, 1999). The effect of these phenotypic changes on carnivorous enemies of herbivores has received most attention (Van Poecke and Dicke, 2004; Turlings and

Ton, 2006). For instance, Lima bean plants that are infested with spider mites start to produce a range of volatiles, including terpenoids and methyl salicylate, several of which attract predatory mites that consume the herbivorous spider mites (Dicke et al., 1990b; Dicke et al., 1999). Moreover, herbivory induces the production of extrafloral nectar in Lima bean plants and this results in increased numbers and duration of visits by e.g. ants and wasps (Kost and Heil, 2005). The increased visitation of the plant by carnivorous arthropods results in a reduced amount of leaf damage (Heil, 2004).

However, plants are at the basis of most terrestrial food webs and consequently they are members of complex communities, both below and aboveground, with a multitude of dynamic, ecological interactions (Price et al., 1980; Dicke and Vet, 1999; Van Zandt and Agrawal, 2004; Bezemer and van Dam, 2005). Ecological interactions may involve direct interactions such as predator–prey interactions or competition among herbivores, as well as indirect interactions such as apparent competition and trait-mediated indirect effects (Holt, 1977; Wootton, 1994; Van Veen et al., 2006; White and Andow, 2006). In many studies species interactions are considered to be fixed: all individuals within a population are considered to have the same characteristics and interact in the same way with other organisms. However, because of phenotypic plasticity, interactions within communities are context-dependent (Agrawal, 2001), which implies that they are not only influenced by genotype, but also by e.g. physiological state, resource availability and interactions with community members. As a result, understanding the effects of phenotypic plasticity is important to understand community dynamics (Figure 2) (Agrawal, 2001).

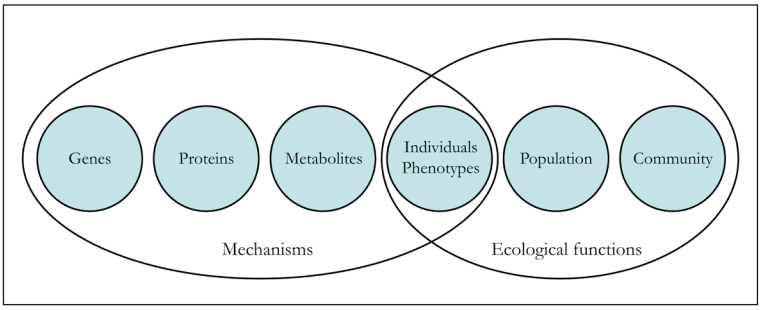


Figure 2. Induced indirect defence can be investigated at different levels of biologcal organisation: by investigation mechanisms of induction at the levels of genes up to indivduals, or by investigating ecological functions at the levels of individuals up to the community. An integration of these two approaches proved to be most rewarding. Phenotypic plasticity plays a central role in understanding both the underlying mechanisms of induced defence and the consequences of induced defence for community dynamics.

Although a phenotypic change may affect many interactions in a community, herbivore-induced indirect plant defences have mostly been studied in a tritrophic context in simple food chains, without taking into account the effects they might have on other community members. Investigating the effects of phenotypic changes on community processes is one of the major challenges that ecologists face in the research on herbivore-induced plant defences (Dicke and Vet, 1999; Kessler and Baldwin, 2001). Understanding the selection pressures and the dynamics

of ecological interactions is important for understanding the ecology and evolution of communities.

To investigate the community consequences of induced indirect plant defences, a thorough understanding of the underlying mechanisms is essential, as mechanistic knowledge allows the development of manipulative tools. Manipulative experiments are important to address the effects of induced defence on a range of individual interactions or on the total set of interactions (Kessler and Baldwin, 2001; Dicke and Hilker, 2003).

In this chapter we will address the mechanisms of induced indirect plant defences and how information on these can be used to investigate the ecological consequences of these defences at the level of multiple ecological interactions and the community.

Induction of indirect plant defense

Herbivores can cause many types of damage to plants, according to the feeding guild to which they belong. For example, caterpillars ingest small sections of the leaves, while others feed on specific parts of the leaf material: leaf-mining insects feed on parenchymal tissue and aphids ingest phloemsap. Different types of damage may result in diverse defence responses in the plant. In this section we will discuss the plant responses and the various mechanisms of their induction.

Response of the plant

In response to arthropod herbivory the plant may activate several major signal-transduction pathways involved in the defence response: the jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) pathways (Dicke and Van Poecke, 2002). These signalling pathways are differentially induced by different feeding guilds or artificial damage (Ozawa et al., 2000; Walling, 2000; Dicke and Van Poecke, 2002; De Vos et al., 2005; Zheng et al., 2007). These signal-transduction pathways also interact: JA can inhibit the effect of SA, and SA can interfere with JA-mediated induction (Peña-Cortes et al., 1993; Sano and Ohashi, 1995). Similarly, JA and ET synergistically affect induction of defence gene expression in tomato (O'Donnell et al., 1996), while ET inhibits the effect of JA on nicotine induction in tobacco (Kahl et al., 2000).

The major signal-transduction pathway involved in plant responses to herbivorous insects is the jasmonic acid or octadecanoid pathway (Table 1). Octadecanoids are synthesised from the 18-carbon fatty acid linolenic acid that is released from membrane lipids in response to stimuli associated with wounding (Narváez-Vásquez et al., 1999). Through the octadecanoid pathway with 13-hydroperoxylinolenic acid, oxo-phytodienoic acid (OPDA), and other compounds as intermediates,

the phytohormone jasmonic acid is produced (Liechti and Farmer, 2002). The members of this pathway have different biological activities. JA induces a large number of genes and the emission of a volatile blend that is similar, though not identical, to the blend induced by herbivory (Dicke et al., 1999; Reymond et al., 2004; De Vos et al., 2005). JA not only affects the induction of defences, but also developmental processes and male fertility (Creelman and Mullet, 1997; Liechti and Farmer, 2002). OPDA also induces gene expression and volatile emission, albeit less effectively as compared to JA (Koch et al., 1999; Stintzi et al., 2001). JA can be converted into methyl-jasmonate or *cis*-jasmone, both of which can induce defences in plants (Farmer and Ryan, 1990; Birkett et al., 2000).

From 13-hydroperoxylinolenic acid there is a side branch of the octadecanoid pathway that leads to the production of so-called green leaf volatiles, such as C6-aldehydes, C6-alcohols, and their acetates

Table 1. The octadecanoid pathway and the effect of manipulation of different steps on volatile emission of Lima bean and the attraction of natural enemies of attackers of Lima bean.

Octadecanoid pathway	Elicitation → / Inhibition --♦	Manipulation with	Volatile emission in response to manipulation	Carnivore attraction
Herbivory				
linolenic acid	→	Linolenic acid	DMNT, TMTT[1]	n.t.
13-hydroperoxylinolenic acid	--♦	Phenidone	Volatile production upon elicitation after pretreatment with inhibitor[1]	n.t.
12,13-epoxy-octadecatrienoic acid	--♦	DIECA	No volatile production upon elicitation after pretreatment with inhibitor[1]	n.t.
12-oxo-phytodienoic acid (OPDA)	--♦	*n*-propyl gallate	No volatile production upon elicitation after pretreatment with inhibitor[1]	n.t.
	→	OPDA	DMNT, TMTT[1,4]	Yes[2]
jasmonic acid (JA)	→	Jasmonic acid	Similar blend as that induced by spider mite infestation[3] Hexenyl acetate, β-ocimene, linalool, DMNT, $C_{10}H_{14}$, $C_{10}H_{16}O$ indole[1]	Yes[2,3]
methyl jasmonate (MeJA)	→	Methyl jasmonate	n.t.	Yes[3]

References: [1]Koch et al. (1999); [2]Dicke and Van Poecke (2002); [3]Dicke et al. (1999); [4]Boland et al. (1999); n.t. = not tested

(Visser and Avé, 1978; Hatanaka et al., 1987). These compounds can attract both herbivorous and carnivorous arthropods on the one hand (Whitman and Eller, 1990; Shiojiri et al., 2006a), and prime neighbouring plants for the induction of defences on the other (Engelberth et al., 2004; Ruther and Fürstenau, 2005). In addition to the octadecanoid pathway, wounding can also induce the hexadecanoid pathway, starting from 7(*Z*),10(*Z*),13(*Z*) hexadecatrienoic acid (Weber et al., 1997; Stintzi et al., 2001) that also leads to JA. The induction of both pathways may result in specific 'oxylipin signatures' that allow plants to fine-tune their responses to wounding or herbivory (Weber et al., 1997). The activation of the signal-transduction pathways can result

in the induction of direct as well as indirect defences. Many types of secondary metabolites involved in direct defence are produced upon herbivore damage, for example non-volatile compounds like proteinase inhibitors, glucosinolates, and alkaloids (Dicke and Van Poecke, 2002). Plants can produce volatiles, such as alcohols, esters and terpenoids or alternative food, such as extrafloral nectar that can function as indirect defence against herbivorous arthropods. Induced plants can produce either the same volatiles as non-induced plants but in different amounts or ratios, or they can produce new volatiles that are not emitted by intact plants (Dicke et al., 1999). However, during both types of responses plants produce the metabolites *de novo*; indicating an active investment in defence, rather than just a passive release of compounds (Donath and Boland, 1994; Paré and Tumlinson, 1997; Mercke et al., 2004).

The contribution of the JA pathway to indirect defence has been well studied in the context of tritrophic interactions. JA in itself is not attractive or deterring, but induces biosynthetic processes in the plant (e.g. the production of volatile infochemicals), that cause behavioural responses of the insects (Avdiushko et al., 1997; Thaler et al., 2002a; Bruinsma et al., 2007, Chapter 3 and 4). JA-induced defence reactions may either deter herbivores, or attract their natural enemies (Gols et al., 1999; Thaler, 1999a; Lou et al., 2005; Bruinsma et al., 2007, Chapter 3 and 4). In addition to JA signalling, tritrophic interactions have been shown to also involve the SA pathway. The production of methyl salicylate (MeSA) is induced by spider mite infestation in e.g. Lima bean and tomato (Dicke et al., 1990b; Dicke et al., 1998; Ozawa et al., 2000), and attracts the natural enemies of the spider mites (De Boer and Dicke, 2004).

Another indirect defence mechanism involving JA signalling is the production of alternative food, such as extrafloral nectar (EFN) that can be used by the members of the third trophic level (Van Rijn and Tanigoshi, 1999; Kost and Heil, 2005; Wäckers and Van Rijn, 2005). EFN is secreted from nectaries outside the flowers, which may occur on the petioles (Koptur, 2005). The nectar composition can differ dramatically between floral and extrafloral nectaries of one plant (Koptur, 2005). The production of EFN increases upon herbivory (Heil et al., 2001; Wäckers et al., 2001). It is highest in leaves where the herbivores are feeding, but can also be increased in systemic leaves (Wäckers et al., 2001). Wounding and JA application increase EFN production. Moreover, EFN secretion is reduced by phenidone, an inhibitor of an early step in the octadecanoid pathway (Table 1) supporting a role for JA in the induction of EFN secretion (Heil et al., 2001). EFN, serving as an alternative food source, may increase the duration of predator visits to plants. Predators disperse more slowly from plants with more EFN as compared to plants with little EFN (Choh et al., 2006), and plants may benefit from the presence of predators.

Induction by herbivores

When herbivores are feeding on a plant, either by leaf chewing, phloem ingestion, or cell content feeding, they induce phytohormone signalling pathways and consequently elicit a plant response. The induced phytohormone signatures are attacker-specific: qualitatively, quantitatively and temporally (De Vos et al., 2005). More and more studies also show the attacker specificity at the level of global gene expression (Voelckel and Baldwin, 2004; Voelckel et al., 2004; De Vos et al., 2005), although other studies recorded quite similar transcriptional responses after attack by different herbivores (Reymond et al., 2004). Herbivore-induced plant volatiles can be specific for herbivore species, and even herbivore instar, feeding on the plant (De Moraes et al., 1998; Takabayashi et al., 2006).

The main groups of plant volatiles induced by herbivory are green leaf volatiles, terpenes, and phenolics. The induction of volatile emission is mediated by the induction of the three main signal-transduction pathways-the JA, SA, and ET pathways-which may be differentially induced by insects from different feeding guilds (Walling, 2000). In Lima bean for example, JA signalling is responsible for the induced production of volatiles in response to caterpillar damage, while both SA and JA mediate the response to spider mite damage (Ozawa et al., 2000). In another plant species, *Medicago truncatula*, caterpillars and spider mites induced qualitatively and quantitatively different volatile patterns. Both JA and SA accumulated in response to damage, but the accumulation differed between the attack by the chewing and by piercing–sucking insects; SA accumulation was higher in response to spider-mite damage compared to caterpillar damage and JA accumulated differently in time for the two modes of attack (Leitner et al., 2005). Likewise, induced changes in gene expression in *Arabidopsis thaliana* show differences between feeding guilds. Five attackers with different modes of attack, ranging from leaf-chewing herbivores to pathogens causing necrotic lesions, showed different degrees of relative induction of the three important signal-transduction pathways (De Vos et al., 2005). In general, it seems that the plant response to phloem-feeding herbivores is more similar to the response to pathogen attack, while leaf-chewing herbivores induce pathways also activated by wounding (Walling, 2000).

Mechanical wounding versus herbivory

When herbivores attack the plant, they inflict physical damage which in itself is sufficient to elicit a subset of the responses to herbivory. Water loss at the wound site may result in osmotic stress and therefore, there is considerable overlap between plant responses to wounding and dehydration. In addition, the plant responds to herbivore-derived compounds present in oral secretions. Physical damage and herbivore-derived elicitors are both responsible for part of the herbivore-induced response of the plant. In many plant species, however, the response to mechanical damage differs from the one elicited by herbivory

(Schoonhoven et al., 2005). This may be partly due to technical difficulties in accurately mimicking herbivory. Mechanical damage differs from herbivore-inflicted damage in the amount of removed tissue, age of tissue, spatial pattern of damage, and timing (Baldwin, 1990). The temporal pattern of mechanical damage was in fact shown to be an important factor influencing its effect (Mithöfer et al., 2005). Yet, when herbivore regurgitant is applied onto the mechanically damaged leaves, the plant response can be similar to the response to herbivory (Turlings et al., 1990; Halitschke et al., 2001). For example, mechanical damage with subsequent regurgitant application induces herbivore-specific plant responses in *Nicotiana attenuata* (Halitschke et al., 2001). Several active compounds have been identified in the oral secretions of feeding insects. For example, the enzyme β-glucosidase and volicitin (a fatty acid-amino acid conjugate, FAC) induce volatile production when added to mechanically damaged cabbage and maize plants, respectively (Mattiacci et al., 1995; Alborn et al., 1997; Schmelz et al., 2001). FACs have been found in all lepidopteran larvae studied to date (Voelckel and Baldwin, 2004). Plants can not only differentiate between mechanical wounding and herbivory, but also between herbivore species, even when they are from the same feeding guild. This has been shown for *N. attenuata*: the plant response to a specialist herbivore differed from the response to two generalist herbivores, and the different responses were correlated with the FAC composition of the regurgitant of the herbivores (Voelckel and Baldwin, 2004).

The difference between induction after mechanical damage and caterpillar feeding has been shown at the gene expression level in *A. thaliana*. Mechanical damage and caterpillar feeding both induce jasmonate-responsive genes and lead to accumulation of JA. However, mechanical damage induces expression of a jasmonate-responsive marker gene PDF1.2, while caterpillar feeding suppresses the induction of this gene. PDF1.2 defence gene induction was suppressed also when caterpillar regurgitant was added to mechanically damaged leaves, indicating a role for caterpillar-derived elicitors in the downregulation of plant defence responses (De Vos, 2006). Three other JA-responsive genes that are induced by caterpillar feeding are not induced by mechanical wounding, demonstrating how herbivory and wounding differentially induce expression of specific genes (De Vos, 2006). In *N. attenuata* plants the endogenous JA levels increase after wounding, and increase even more when oral secretion is applied to the wounds. The same pattern can be observed for ethylene emission in these plants: punctured plants treated with oral secretion temporarily emit more ethylene than do punctured plants treated with water, while ethylene emission upon herbivory increases for as long as herbivory continues (Kahl et al., 2000).

Priming

Apart from direct upregulation of defence signalling cascades or gene expression, the ability of the plant to rapidly activate cellular defence

responses can be enhanced, a process that is called priming (Conrath et al., 2002). When exposed to a priming stimulus the plant does not respond with the immediate production of defence compounds, but rather enters a sensitised state allowing it to respond faster or stronger to subsequent challenges in the future (Turlings and Ton, 2006). Priming has originally been demonstrated for plant–pathogen (Conrath et al., 2002; Conrath et al., 2006) and plant–rhizosphere bacteria interactions (Verhagen et al., 2004) and appears to be advantageous with respect to defence-associated metabolic costs.

Priming can occur in response to different stimuli, such as plant volatiles, pathogen infestation or herbivory. Priming of indirect defences has been shown for induced volatile emission as well as EFN secretion. Exposure to green leaf volatiles from neighbouring damaged plants results in higher endogenous JA levels and higher emission of volatiles upon herbivory or mechanical damage compared to non-exposed plants (Engelberth et al., 2004). Also exogenously applied single compounds, such as (Z)-3-hexenal, (Z)-3-hexen-1-ol, and (Z)-3-hexenyl acetate, can prime plants for responses to herbivore attack (Engelberth et al., 2004). Recently, a field study demonstrated the priming of EFN secretion. Lima bean plants were exposed to an artificial volatile blend mimicking the volatile emission from herbivore-induced Lima bean plants. Upon wounding, these plants secreted more EFN as compared to non-exposed controls indicating a priming effect of the exposure to volatiles (Heil and Kost, 2006).

Responses of community members to induced indirect plant defence

Induced infochemicals (sensu Dicke and Sabelis, 1988), once released from the plant, can be exploited by any of its community members, including neighbouring plants, herbivores, predators, parasitoids, pollinators and other community members, both above- and belowground (Figure 3). The infochemicals can function as a direct defence by repelling herbivores. For example, butterflies avoid oviposition on plants that are induced by either the presence of eggs or feeding damage (Rothschild and Schoonhoven, 1977; Landolt, 1993; De Moraes et al., 2001). High levels of direct defence may deter herbivores from feeding or ovipositing on a plant, and may also slow down development of larvae, rendering them more vulnerable to natural enemies (Rothschild and Schoonhoven, 1977; Stout and Duffey, 1996; Thaler et al., 1996). However, direct and indirect defence mechanisms can act antagonistically. Plant toxins that are ingested by the herbivore may be sequestered to affect the development of carnivores, or negatively influence carnivore fitness due to compromised host size or quality (Ode, 2006). Natural populations of Senecio jacobaea exhibit genetic variation of pyrrolizidine alkaloid concentration. Plants infested by aphids and ants that tend the aphids

have lower PA-levels than plants without aphids and their ant tenders. The ants can protect the plant from complete defoliation, thus benefiting the plants in years with high pressure from the specialist herbivore Tyria jacobaeae, while in years with low T. jacobaeae abundance these plants may suffer more fitness costs from aphid herbivory than plants with higher PA-levels (Vrieling et al., 1991). The effects of induction on other community members than herbivores and the complexity of their interactions are discussed in the following paragraph.

Responses of members of the third trophic level

Plants can benefit in terms of fitness gain from carnivores that attack the herbivores (Van Loon et al., 2000; Fritzsche Hoballah and Turlings, 2001). Herbivores, however, are under selection to be inconspicuous, and are small in comparison to the plants they are feeding on. Consequently, carnivorous arthropods often depend on plant cues to locate their herbivorous victims (Turlings et al., 1990; Steinberg et al., 1992; Vet and Dicke, 1992; Geervliet et al., 1994). Even though host-derived stimuli are potentially more reliable for host location, their use is often limited by low detectability, especially at longer distances (Vet and Dicke, 1992). Therefore, carnivorous arthropods are usually more strongly attracted by plant-derived volatiles as compared to volatiles derived from their herbivorous victims (Turlings et al., 1990; Turlings et al., 1991; Steinberg et al., 1993; Geervliet et al., 1994; Dicke, 1999). Attraction by volatiles from host-infested plants and by EFN was shown for egg- as well as larval parasitoids and predators (e.g. Blaakmeer et al., 1994a; Geervliet et al., 1997; Lou et al., 2005; Choh et al., 2006; Hilker and Meiners, 2006; Mumm and Hilker, 2006). Induced levels of EFN also increase the abundance of ants, wasps and flies (Kost and Heil, 2005), and reduce the amount of leaf damage (Heil, 2004).

The major signal-transduction pathway involved in attraction of natural enemies seems to be the JA-pathway (Dicke and Van Poecke, 2002). The involvement of JA in induced attraction of members of the third trophic level has been demonstrated both by manipulation of JA on the level of the plant's phenotype and the plant's genotype. JA-treated plants attract natural enemies of herbivorous arthropods and have increased parasitism rates in field (Gols et al., 1999; Thaler, 1999a; Ozawa et al., 2004). Moreover, jasmonate-deficient plants are less attractive to natural enemies than control plants when attacked by herbivores (Thaler et al., 2002a).

Responses of members of higher trophic levels

The enemies of herbivores can in turn fall victim to members from higher trophic levels, for example to hymenopterous hyperparasitoids, also called secondary parasitoids (Brodeur, 2000). Induced changes in plant chemistry affect not only the development and survival of herbivores and their parasitoids, but also that of secondary parasitoids (Harvey et al., 2003). Performance of *Lysibia nana*, a hyperparasitoid with a broad host range, was shown to be negatively affected by high

levels of defensive toxins in the host plant (Harvey et al., 2003; Soler et al., 2005). Secondary parasitoids are often compared to primary parasitoids, since both share common life-history strategies (Brodeur, 2000). However, as compared to primary parasitoids, little is known about their host searching strategies and whether or not they use plant volatiles to locate (parasitised) herbivores (Harvey et al., 2003; Buitenhuis et al., 2005). Plant-derived cues may be of limited value for hyperparasitoids for several reasons: firstly, although herbivore-induced plant volatiles may be reliable cues for primary parasitoids, they do not guarantee the presence of a parasitised host for the secondary parasitoid. Secondly, primary parasitoids are often more specialised than secondary parasitoids (Buitenhuis et al., 2005). Yet, a recent study demonstrated that primary parasitoids are in fact able to discriminate between herbivore-induced plant volatiles emitted from plants damaged by unparasitised or parasitised caterpillars (Fatouros et al., 2005b), suggesting that reliable plant cues may be available to secondary parasitoids as well.

Several studies have been conducted on host searching behaviour of secondary parasitoids with varying results. A specialised ectoparasitoid *Euneura augarus* relies on plant volatiles for long range searching (Völkl and Sullivan, 2000). However, *E. augarus* does not distinguish between plants with and without host mummies for long range searching. The two hyperparasitoid species *Alloxysta victrix* and *Dendrocerus carpenteri* were attracted to herbivore-induced volatiles [oat plants infested with *Sitobion avenae* aphids (Siri, 1993)], while in another study with another plant–host–primary parasitoid system (potato infested with *Macrosiphum euphorbiae*) they were not (Buitenhuis et al., 2005). Buitenhuis et al. (2005) did find analogies in foraging behaviour of primary and secondary parasitoids in the use of contact cues while searching on a plant. Host searching behaviour of hyperparasitoids may depend more on contact cues and less on plant volatiles compared to primary parasitoids (Buitenhuis et al., 2005).

Responses of pollinators

Sexual reproduction of many plant species depends on pollination by honey bees, bumble bees, solitary bees, syrphid flies or moths (Klein et al., 2007). Herbivory in early stages of plant growth reduces the photosynthetic area of the plant, and may result in smaller plants and a shorter flowering period. This is possibly due to allocation of resources to defences, rather than growth and reproduction (Poveda et al., 2003). Herbivory may affect the production of pollen and nectar, the quality of nectar, morphology of flowers and may reduce seed production (Lehtilä and Strauss, 1997; Hambäck, 2001; Poveda et al., 2003). Both nectar quality and quantity are parameters that determine the number and type of pollinators that are attracted to the plants (Potts et al., 2003). While extrafloral nectar is known to increase after herbivory, it remains unknown whether herbivory also affects floral nectar production (Adler et al., 2006). Just a handful of studies have addressed the effect of herbivory on floral chemistry. Leaf herbivory on tobacco plants

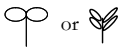
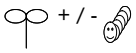
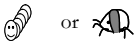
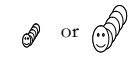
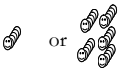
increased alkaloid concentration in the nectar (Adler et al., 2006), while other studies have shown increases in defence compounds such as nicotine and glucosinolates in flower tissues (Euler and Baldwin, 1996; Ohnmeiss and Baldwin, 2000; Strauss et al., 2004; Smallegange et al., 2007). It is interesting to note that in tobacco plants nicotine levels in the corolla are lower during the scotophase, when moths are attracted for pollination (Euler and Baldwin, 1996).

However, as yet there is little knowledge on the influence of herbivory on pollination. Several studies have reported an indirect effect of herbivory on pollination. Herbivory may affect flowering traits, for example reducing the number of flowers, flower size or plant height. This in turn, can affect flower visitation by pollinators (Lehtilä and Strauss, 1997; Adler et al., 2001; Hambäck, 2001). Lehtilä and Strauss (1997) showed that bees prefer undamaged radish plants over damaged plants, but this difference could be explained by reduced flower size and number. However, syrphid flies preferred undamaged plants over damaged ones, even when the plants were controlled for flower size and number; indicating a chemical basis for syrphid fly attraction. While in this case pollinator attraction decreased, herbivory can also enhance pollinator attraction. Root herbivory for example, can increase flower visitation, as demonstrated in mustard plants by Poveda et al. (2003; 2005). Although these examples demonstrate the influence of herbivory on pollination, the mechanism behind this phenomenon is not yet elucidated. Possibly the effect on plant growth, number of flowers, plant volatiles or a change in nectar quantity or quality, determine the change in attraction of pollinators.

Responses of neighbouring plants

Plants may gain a fitness benefit from responding to herbivore-induced volatiles from neighbouring plants that are being attacked. Such a signal is indicative of the imminent danger, and the receiving plant may profit from exploiting this information by readying its defence (Dicke and Bruin, 2001). Herbivore-induced plant signals can be exploited by neighbouring plants of the same and of different species alike (Engelberth et al., 2004; Baldwin et al., 2006). Strong signals may immediately activate plant defences, while lower (more common in nature) concentrations of signalling molecules can prime the plant for attack (Turlings and Ton, 2006). This was nicely shown in a field study by Kessler et al. (2006) reporting that plant volatiles from damaged sagebrush plants can prime the induction of proteinase inhibitors in nearby tobacco plants. The primed tobacco plants received less damage from subsequent herbivore attack as compared to non-exposed control plants. Not only volatile emission, but also EFN secretion can be primed by exposure to volatiles from damaged plants. Plants that have been exposed to volatiles from herbivore-infested conspecific plants produce more EFN when they

Table 2. Plant volatile emission can be highly specific in response to different stimuli. This table non-exhaustively illustrates the specificity of plant volatile emission and the subsequent perception and discrimination between signals by insects.

Plant volatiles differ depending on:	**System**	**Result**	**References**
Plant species 	Tobacco, cotton and maize – *Heliothis virescens* and *Helicoverpa zea* – parasitic wasp *Cardiochiles nigriceps*	All 3 plant species emit different volatile blends in response to their attackers.	De Moraes et al. (1998)
Plant cultivar 	Maize cultivars and wild relatives	The plant cultivars emitted qualitatively and quantitatively different volatile blends	Gouinguené et al. (2001)
Plant with or without host 	Maize *Zea mays* – beet armyworm *Spodoptera exigua* – *Cotesia marginiventris*	Undamaged and herbivore-damaged plants differ in volatile emission and *C. marginiventris* can discriminate between the odour blends.	Turlings et al. (1990)
	Pine – aphids – *Cinara pinea*– hyperparasitoid *Euneura augarus*	Hyperparasitoid cannot discriminate at long range between plants with and without aphid mummies	Völkl and Sullivan (2000)
Herbivore species 	Tobacco, cotton and maize – *Heliothis virescens* and *Helicoverpa zea* – parasitic wasp *Cardiochiles nigriceps*	Plants produce herbivore-specific chemical signals and *C. nigriceps* females prefer host-infested over non-host-infested plants	De Moraes et al. (1998)
	Bean plants *Vicia faba* – host: pea aphid *Acyrthosiphon pisum*, non-host black bean aphid *Aphis fabae* – parasitoid *Aphidius ervi*	Host and non-host aphid induce different volatiles, *A. ervi* can discriminate between host-infested and non-host-infested plants	Du et al. (1996; 1998)
Herbivore instar 	Maize *Zea mays* – common armyworm *Pseudaletia separata* - *Cotesia kariyai*	*C. kariyai* discriminates between undamaged plants and plants damaged by 1st to 4th instar larvae, but not by later instar larvae. And early and late instar larvae induce qualitatively and quantitatively different volatile blends	Takabayashi et al. (1995)
Local or systemic induction 	Lima bean *Phaseolus lunatus* – spider mites *Tetranychus urticae* – predatory mites *Phytoseiulus persimilis*	Undamaged leaves of a spider mite-infested plant are induced to emit volatiles that attract the predators	Dicke et al. (1990a)
Time since induction 	Brussels sprouts *Brassica oleracea* – large cabbage white *Pieris brassicae* – *Cotesia glomerata*	Brussels sprouts plant becomes attractive to *C. glomerata* 30 minutes after caterpillar infestation, reaches a maximum after 3 hours and then remains constant for at least 14 hours	Scascighini et al. (2005)
Time of day 	Tobacco *Nicotiana tabacum* – moth *Heliothis virescens*	Difference in volatile emission during day and night, influences night-active herbivores	De Moraes et al. (2001)
Infestation rate 	Field elm *Ulmus minor* – elm leaf beetle *Xanthogaleruca lutteola* –	Twigs with a low infestation rate are more attractive to beetles than uninfested or heavily infested twigs	Meiners et al. (2005)

are attacked themselves (Heil and Kost, 2006), although this effect was only observed during the early stages of herbivore attack (Choh and Takabayashi, 2006).

Above- and belowground interactions

Most studies on induced defences have focused on aboveground interactions. However, belowground interactions can have an important effect on aboveground processes. For example, root herbivory and belowground plant mutualists, such as arbuscular mycorrhizal fungi, affect pollination (Poveda et al., 2003;2005; Wolfe et al., 2005). Processes similar to those reported to occur aboveground do also take place belowground. Plants can release volatiles in response to root herbivory that attract natural enemies of the root herbivores. This was first reported for a coniferous host plant, *Thuja occidentalis*, that when exposed to weevil larvae, releases chemicals attractive to nematodes that can parasitise the larvae (Van Tol et al., 2001). Rasmann et al. (2005) showed the same phenomenon for maize plants, and identified a chemical attracting the entomopathogenic nematode *Heterorhabditis megidis*, a natural enemy of the attacking beetle. Plant-to-plant signalling also takes place belowground. Plants emit belowground signals that are exploited by neighbouring plants for the attraction of predators (Chamberlain et al., 2001; Dicke and Dijkman, 2001), or by parasitic plants to induce germination (Bouwmeester et al., 2003; Runyon et al., 2006).

In addition to belowground interactions, it is important to consider the interactions between above- and belowground communities. Cotton plants, for example, increase aboveground extrafloral nectar production upon attack by root-feeding wireworms (Wäckers and Bezemer, 2003). Root herbivory, as well as arbuscular mycorrhizal fungi, can also change aboveground volatile emission, and subsequently increase the number of visits by parasitoids of aboveground herbivores (Masters et al., 2001; Neveu et al., 2002; Guerrieri et al., 2004; Soler et al., 2007). The performance of the aboveground multitrophic community associated with a plant differs between plants with and without root herbivory (Soler et al., 2005). Root herbivory changed plant quality, which in turn negatively affected the performance of an aboveground herbivore, a primary parasitoid and secondary parasitoid. Bezemer et al. (2005) found that the performance of aphids was reduced by the presence of nematodes or micro-organisms, while the primary aphid parasitoids were positively affected by these belowground community members. These studies illustrate that soil communities can influence interactions and performance of aboveground species at multiple trophic levels. To our knowledge, no such studies have been conducted to investigate possible effects of aboveground herbivory on attraction and performance of belowground carnivores.

Indirect defence in a complex and variable world

Induced plant volatile blends can be very specific: parasitoids and predators can distinguish between induced blends from different plant species, herbivore species (or even herbivore instars), between feeding- and egg-induced responses, and between local and systemic damage (Table 2). While most studies have examined simple tritrophic systems consisting of one plant, one herbivore and one of its natural enemies, in the field an organism interacts with many more species. Host plants are frequently infested by more than one herbivore (e.g. Vos et al., 2001). For community dynamics it is important whether the organisms receiving plant-emitted signals are able to distinguish between signals indicating the presence of their host from non-host signals and other background odours. Shiojiri et al. (2002) compared the host searching behaviour of two species of parasitoid wasps on host plants infested with one or two herbivores. One of the parasitoids, *Cotesia plutellae*, was more strongly attracted to plants infested solely with its host, while the other parasitoid, *Cotesia glomerata*, preferred plants with both herbivores (Shiojiri et al., 2001). Consequently, a plant infested with both herbivores provides an enemy-free space for *C. plutellae*'s host (i.e., *Plutella xylostella*), and an enemy-dense space for *C. glomerata*'s host (i.e., *Pieris rapae*). The oviposition preference of the adult herbivores corresponded to this pattern; while *Pl. xylostella* preferred plants with both herbivores, *P. rapae* did not show any preference (Shiojiri et al., 2002). In this example, *Pl. xylostella* profits from a positive indirect effect (associational resistance) through the presence of *P. rapae* (White and Andow, 2006). Defence responses to herbivores may also interfere with other interactions in the community, such as pollination. For tobacco plants there is a trade-off between repelling herbivores and attracting pollinators. As briefly mentioned previously, these plants reduce the level of toxins and increase emission of a pollinator attractant by their flowers in the evening. This way tobacco can defend itself against herbivory during daytime and attract pollinators at night (Euler and Baldwin, 1996).

While some community members are able to distinguish between different volatile blends, others cannot, or only after associative learning (Takabayashi et al., 2006). Several arthropod species, such as *Phytoseiulus persimilis*, *Cotesia marginiventris* and *C. glomerata*, can learn to associate certain odours with a reward (Geervliet et al., 1997; Turlings and Fritzsche, 1999; De Boer et al., 2005). Learning to associate odours with the presence of host or prey may be a way to cope with the variation in volatile blends. Learning new associations may take some time and multiple experiences. In the meantime the host or prey species may profit from its 'invisibility' to its natural enemy while the parasitoid or predator wastes time on searching on plants without its host or prey (Shiojiri et al., 2002). The temporal pattern in learning to respond to certain blends and losing this learned response will affect the risk of herbivores to fall victim to their enemies. Temporary refuges may stabilise

predator–prey or parasitoid–host systems and thus affect dynamics of arthropod communities on plants (Vos et al., 2001; Takabayashi et al., 2006). To gain more insight into these subtle interactions and ecosystem stability, field studies in which one component is changed could provide an important tool to investigate the impact on the community.

Manipulation of indirect plant defence

To investigate the effect of indirect defences and their components, a manipulative approach is most rewarding. Manipulation of defence responses can provide information on both the mechanisms of plant defence and the ecological consequences of changes in the plant's phenotype. A range of manipulative approaches can be taken that are explained below.

Perfuming with individual compounds

A simple change in a plant's phenotype can be made by adding a single compound to an undamaged plant. This method allows testing the importance of individual compounds for attraction of, for example, predators and parasitoids to the plant against a background of natural odours (Dicke et al., 2006). Even though carnivorous arthropods are usually attracted to complex odour blends, increased attraction of carnivores due to addition of individual compounds has been recorded with this method (De Boer and Dicke, 2004). Also in a field study, the addition of linalool to *N. attenuata* plants resulted in both increased predation of herbivore eggs and larvae, and decreased oviposition by an herbivore (Kessler and Baldwin, 2001).

Fractionation and filtering

Phenotypic manipulation of the volatile blends can also be accomplished by using filters to collect compounds with specific chemical properties selectively (D'Alessandro and Turlings, 2006), or by fractionation of the headspace and subsequently testing different fractions of the blend for biological activity (Turlings and Fritzsche, 1999; Van den Boom, 2003). As compared to the testing of single compounds, these methods have the advantage of conserving the ratios of different volatiles as they are present in natural herbivore-induced volatile blends. Filters can be used to remove one or more compounds, which are suspected to show biological activity, from the total volatile blend. It is then possible to test their importance for animal behaviour in olfactometer experiments (D'Alessandro and Turlings, 2006). With this method D'Alessandro and Turlings (2005) showed that the selective removal of the more polar plant volatiles using silica filters decreases the attractiveness of the volatile blend to a parasitoid wasp, *Cotesia marginiventris*, but not to another parasitoid *Microplitis rufiventris*. This study illustrates how filtering can be used as a tool to find the ecologically relevant volatiles for different species on all trophic levels using various adsorbing materials.

For fractionation, the mixture of defence compounds can be trapped onto an absorbent material and subsequently recovered by chemical or thermal desorption. Subtractive fractionation and testing of the different fractions for their biological activity provides a useful tool to identify compounds involved in indirect defence (Van den Boom, 2003).

Genetic modification

Another way to manipulate induced defences of plants, is by genetic modification of signal-transduction or biosynthetic pathways. Carefully modified plants that differ in a single gene can be compared with wild-type plants to gain more insight into mechanisms of induced defence and the resulting ecological effects (for review, see Snoeren et al., 2007). Otherwise identical plants can be induced by herbivory and the differences between the wild type and mutant can be studied at the levels of gene expression, volatile production and response of the plant-associated community. The approach is limited by the availability of modified plants. For some species like *A. thaliana* however, a wide variety of mutant and transgenic plants is available. Genetic modification is an excellent tool to study the ecological relevance of individual compounds that are difficult to obtain synthetically. For example, undamaged *A. thaliana* plants transformed with a terpene synthase from strawberry, produce the terpenoids 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) and (*E*)-nerolidol which results in the attraction of predatory mites (Kappers et al., 2005). However, *A. thaliana* has its limitations, such as its very early phenology and consequently limited interaction with potentially associated organisms, as a model for community ecology studies.

Transgenic *N. attenuata* plants that have been modified in the octadecanoid signal-transduction pathway received more herbivory and by more herbivore species than wild type control plants (Kessler et al., 2004). Similarly, the tomato mutant *def-1* is deficient in JA accumulation through a mutation early in the JA pathway. As a result, the plants produce an incomplete volatile blend in response to herbivore damage, and the natural enemies of the herbivores do not discriminate between volatile blends from induced and non-induced plants (Thaler et al., 2002a; Ament et al., 2004). However, for many ecological model species mutants or genetically modified plants are not (yet) available.

Chemical elicitors and inhibitors

Signal-transduction and biosynthetic pathways can be manipulated through the application of (specific) inducers or inhibitors. By artificially inducing or inhibiting different steps of the signalling pathways it is possible to study ecological interactions at different trophic levels, or in the whole community. Plant-emitted volatile blends can be manipulated by interfering with the signal-transduction pathways, and the importance of individual steps can be investigated by specifically activating or blocking individual pathway enzymes. When these changed blends are

offered to (members of) the insect community, this will provide insight into the ecological relevance of these pathways.

The most extensively tested elicitors to date are the phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA). These elicitors were shown to play an important role in induced defence in many plant species against a wide range of herbivores. JA induces volatile blends similar to those induced by herbivore damage (Hopke et al., 1994; Dicke et al., 1999; Ozawa et al., 2000; Ozawa et al., 2004), and while herbivores are negatively influenced (Van Dam et al., 2000; Thaler et al., 2001; Bruinsma et al., 2007, Chapter 3), carnivorous arthropods are attracted to plants that have been induced with JA or MeJA (Thaler, 1999a; Van Poecke and Dicke, 2002; Gols et al., 2003; Chapter 4 and 5). For many other elicitors and phytohormones, such as methyl salicylate, linolenic acid, OPDA, β-glucosidase, cellulysin, and alamethicin, similar studies demonstrated their effects on plants and community members (Mattiacci et al., 1995; Koch et al., 1999; Dicke and Van Poecke, 2002; De Boer and Dicke, 2004; Ozawa et al., 2004; Chapter 6). One of the advantages of using elicitors is the possibility to apply a controlled dose to specific plant parts, whereas it is practically impossible to control the amount of injury inflicted by insects or other biotic agents. However, it is not easy to relate the externally applied dosage to intracellular concentrations and effects.

Specific inhibitors, although not used quite as extensively as elicitors, are suitable to demonstrate the importance of different steps in signal-transduction pathways as well. However, accumulation of pathway intermediates just before the inhibited step may cause physiological side effects. Making use of inhibitors, Koch et al. (1999) showed the importance of early steps in the octadecanoid pathway for volatile emission of Lima bean (Table 1). Phenidone for example, inhibits the activity of lipoxygenase, which results in incomplete volatile blends (Piel et al., 1997; Koch et al., 1999) and reduced EFN secretion (Heil et al., 2004), indicating the importance of this early step in the octadecanoid pathway for indirect defence responses.

From the lab to the field

To gain insight into the ecology of induced defences, field studies incorporating the complexity of natural ecosystems are indispensable. Manipulation through one of the methods described above may facilitate research on the effects of induced indirect defence on interactions between community members and the stability of the system.

Most studies addressing induced plant defence are laboratory studies, studying simple systems of one plant, one herbivore and its natural enemies. This provides detailed insight into the effects of induced

defences on individual interactions. Through greenhouse and semi-field studies with more complex set-ups, for instance by introducing background odours under controlled conditions (Janssen, 1999; Dicke et al., 2003a), more insight will be gained in field situations. However, in order to use knowledge gained from these studies, the relative importance of these pieces of information should be assessed in the field. Also biological control in agricultural fields may benefit from such knowledge and understanding of multitrophic interactions in the field.

Natural ecosystems

For studying the ecology of herbivore-induced plant responses, it is necessary to address natural ecosystems. For instance, in wild radish induced responses to herbivory increase plant fitness in natural environments (Agrawal, 1999). Herbivory increases trichome density and subsequently reduces preference and performance of several herbivores. The effectiveness of induced defences is clear from a field study on *N. attenuata* where herbivory was reduced by as much as 90% by the addition of defence compounds (Kessler and Baldwin, 2001). In a later study Kessler et al. (2006) showed that volatiles from damaged sagebrush can prime responses in *N. attenuata* and reduce herbivore damage to the exposed plants. However, despite the use of natural populations of *N. attenuata*, the ecological relevance of this study is questionable because the sagebrush that was used for elicitation occurs in a different successional stage than *N. attenuata*. Furthermore, the effect was only detected at a short range, when the plants grew within 15 cm from each other.

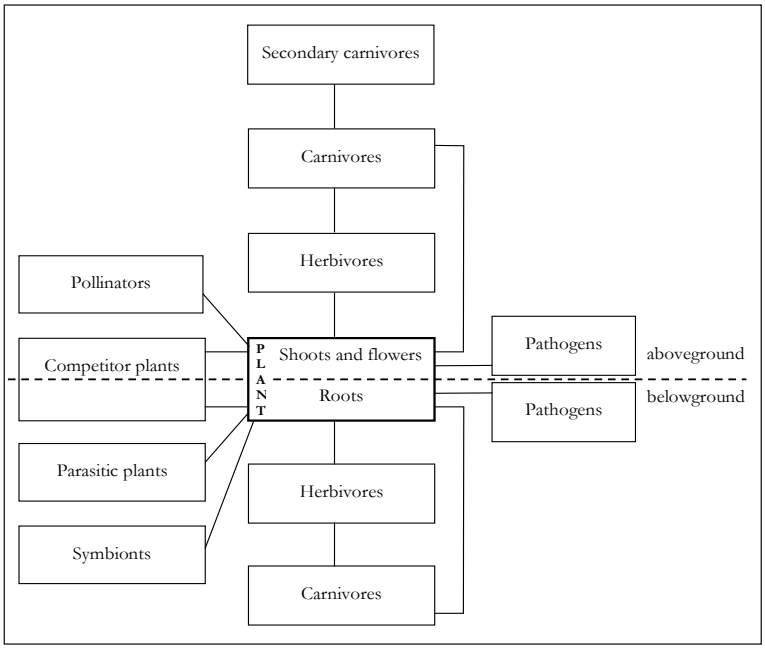


Figure 3. Schematic view of the complexity of interactions above- and belowground in a multitrophic community (pathogens of herbivores, carnivores and pollinators have not been included).

In naturally occurring milkweed populations early-season herbivory affects subsequent herbivory throughout the season, thereby affecting community structure (Van Zandt and Agrawal, 2004). In a natural community, Agrawal (2004) studied the complex interactions between milkweed and competing grass, in presence or absence of root and leaf herbivory. He concluded that the genetic differences, competition, and herbivory resulted in complex interactions that may result in diffuse co-evolution between milkweed and its herbivores. To improve our understanding of the evolution of induced defences and resistance of herbivores more field studies are needed.

The use of elicitors in the field can provide information on the ecological relevance of pathways and certain steps therein. For example, the application of JA to tomato plants in the field increased parasitism of herbivores and thus showed the involvement of JA-induced changes in the attraction of carnivores (Thaler, 1999a). The application of MeJA to tobacco plants demonstrated the costs of jasmonate-induced responses. In environments with herbivore pressure, induced plants suffered less from herbivore attack and produced more viable seeds than non-induced plants. However, undamaged plants produced more seeds when they were not induced compared to jasmonate-induced plants (Baldwin, 1998).

Agricultural systems

Induced plant defences can aid pest control in agricultural systems. Attracting natural enemies of herbivores to crops can help control pests in agriculture; in the field as well as in greenhouses (Dicke et al., 1990a; Turlings and Ton, 2006). Therefore, understanding the mechanisms involved in plant defences and the consequences for the community associated with the plant can aid crop protection. Manipulation may increase the effectiveness of plant defences, by attracting natural enemies before considerable damage is done by herbivores and by deterring oviposition by herbivores. This can be achieved by inducing the plant with phytohormones like cis-jasmone or jasmonic acid (Thaler, 1999a; Birkett et al., 2000; Heil, 2004). Another possibility is the use of genetically modified crops that produce volatile blends that are more attractive to predators than genotypes currently used. The technology is being developed (Kappers et al., 2005; Schnee et al., 2006). However, the consequences of genetically modified plants for the community, above- as well as belowground, and the effect on interactions between different community members still need to be addressed (Groot and Dicke, 2002; Kowalchuk et al., 2003).

A lot of the research on genetic modification of plant defence has been done in the model plant A. thaliana, for which many mutants are available (Turlings and Ton, 2006). Knowledge about *A. thaliana* can be extrapolated to *Brassica* species, and therefore readily be applied in agricultural settings with crop species like *Brassica oleracea* or wild *Brassica* species (Broekgaarden et al., 2007; Zheng et al., 2007). In

conclusion, the step from the laboratory into the field has not been made often yet. It will be important to make this step for different plant species to gain insight into the effects of induced indirect defence on community processes.

Perspectives

The effects of induced defence-related phenotypic changes in plants on community dynamics are difficult to predict, because many aspects are involved and the variability of plant responses is enormous. While many bi- and tritrophic interactions are well studied, plants in nature are usually under the attack of a range of organisms at the same time. How this affects plant defence has only just begun to be addressed and first results show that the effects may be an increase as well as a decrease in defence intensity (e.g. Dicke et al., 2003a; Rodriguez-Saona et al., 2005; Cardoza and Tumlinson, 2006).

Although most studies thus far have focused on aboveground processes, the influence of the changes in plant phenotype is not limited to the aboveground community. Aboveground interactions can change belowground root exudates and influence the soil community, and belowground damage can influence aboveground indirect defence (Bezemer and van Dam, 2005). The reverse, however, i.e. the effect of aboveground interactions on belowground indirect defence, remains as yet uninvestigated (Bezemer and van Dam, 2005). Incorporating these interactions in future studies will greatly enhance our insight into the effects of induced indirect defence on the functioning of complex communities. In addition to plant–pathogen and plant–herbivore interactions, plants may also be under the attack of parasitic plants (Bouwmeester et al., 2003; Runyon et al., 2006), or interact with belowground symbiotic organisms such as mycorrhizal fungi or symbiotic bacteria (Gange et al., 2002). Furthermore, aboveground endophytic organisms can influence the plant's defensive phenotype and consequently also the interactions with community members (Omacini et al., 2001). Incorporating these interactions in the investigations of indirect defence of plants in a community ecology approach will increase complexity, yet doing so is essential to gain a meaningful understanding of the effects of indirect plant defence on plant ecology.

Another area of research that has not received a lot of attention so far is the searching behaviour of members of the higher trophic levels, such as hyperparasitoids. How they find their host and whether they use plant cues remains largely unknown (Buitenhuis et al., 2005). The same applies for pollinators. Though some effects of herbivory on pollination have been reported (Lehtilä and Strauss, 1997; Poveda et al., 2003), the underlying mechanisms remain to be unravelled and which

signal-transduction pathways are important in this respect, waits to be investigated.

For a complete understanding of the ecology and evolution of communities, it is necessary to include all trophic levels in field studies. Manipulative studies are likely to provide the best way forward. They can be used in the laboratory to investigate individual interactions and are a valuable tool to investigate the effects of induced defences on the community in the field (Kessler and Baldwin, 2001; Kessler et al., 2004). Using an integrated approach with molecular, chemical, and behavioural methodology will significantly advance the research in this area (Baldwin et al., 2001).

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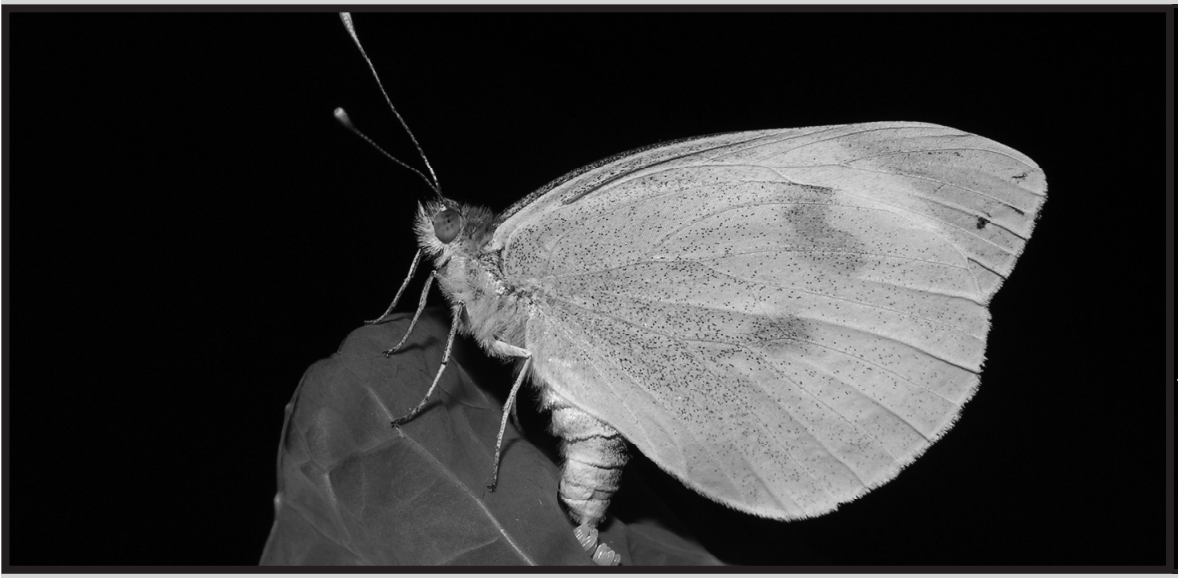

Chapter 3

HANS SMID

Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores

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Abstract

Jasmonic acid (JA) is known to be a key hormone involved in plant defence responses. The effect of JA-treatment of cabbage plants on their acceptability for oviposition by two species of cabbage white butterflies, *Pieris rapae* and *P. brassicae*, was investigated. Both butterfly species laid fewer eggs on leaves of JA-treated plants compared to control plants. We show that this is due to processes in the plant after JA-treatment rather than an effect of JA itself. The oviposition preference for control plants is adaptive, as development time from larval hatch until pupation of *P. rapae* caterpillars was longer on JA-treated plants. Total glucosinolate content in leaf surface extracts was similar for control and treated plants; however, two of the five glucosinolates were present in lower amounts in leaf surface extracts of JA-treated plants. When the butterflies were offered a choice between the purified glucosinolate fraction isolated from leaf surface extracts of JA-treated plants and that from control plants, they did not discriminate. Changes in leaf surface glucosinolate profile therefore do not seem to explain the change in oviposition preference of the butterflies after JA-treatment, suggesting that as yet unknown infochemicals are involved.

Introduction

Plants can be attacked by many herbivorous insects and have evolved a variety of defence strategies, including morphological barriers, synthesis of toxic or repellent secondary metabolites, and the release of synomones that attract natural enemies of the herbivores. These defences can be constitutive, i.e., expressed independent of the presence of an attacker, or inducible, in which case defence compounds accumulate in response to attack (Karban and Baldwin, 1997). Herbivores can detect induced defensive compounds and respond by avoiding these plants which signal lower suitability as a host plant (Landolt, 1993; De Moraes et al., 2001; Kessler and Baldwin, 2001; Meiners et al., 2005). Induced plant defence can affect herbivorous insects directly through the production of toxic compounds, or indirectly through the production of cues that indicate intra- or interspecific competition for the herbivores (Schoonhoven et al., 2005). Moreover, induced plant defence signals can reduce the enemy-free space for the herbivores. For parasitoids and predators induced infochemicals may indicate the presence of their host or prey on the plant (Turlings et al., 1990; Dicke and Vet, 1999). Phenotypic changes in individual plants may therefore affect insects at different trophic levels and, thus, the composition of the insect community and food web associated with the plant (Price et al., 1980; Van Zandt and Agrawal, 2004; Takabayashi et al., 2006).

Already in the 19th century Kirby and Spence (1863) observed that *Pieris brassicae* females preferred to lay their eggs on plants devoid of eggs. Later this was confirmed under more controlled conditions by Rothschild and Schoonhoven (1977) for both *P. brassicae* and *P. rapae*. This avoidance of infested plants is caused by a physiological response of the plant to oviposition, rather than by compounds excreted by the butterflies themselves (Blaakmeer et al., 1994b). *Pieris brassicae* also avoid egg deposition on leaves with feeding larvae (Rothschild and Schoonhoven, 1977). It was postulated that butterflies avoid laying eggs on herbivore-infested plants because herbivore attack induces defence compounds in plants that can influence the performance of their offspring and to reduce the risk of inter- or intraspecific competition and parasitism (Thompson and Pellmyr, 1991; Shiojiri et al., 2002). Egg-induced chemical changes in *Brassica* plants are also known to arrest *Trichogramma* parasitoids that parasitise *Pieris* eggs (Fatouros et al., 2005a).

Oviposition-site selection involves an important behavioural decision in the life cycle of a herbivorous insect because hatching larvae have limited dispersal capacity (Renwick and Chew, 1994). *Pieris rapae* is a solitary butterfly that lays one egg at a time, whereas *P. brassicae* is gregarious and lays batches of about 20-100 eggs. *Pieris rapae* appears to spread the risk of larval mortality, laying few eggs within any patch. This has the advantage of being able to exploit isolated plants (Davies and Gilbert, 1985). *Pieris brassicae*, however, needs patches of plants, because one large egg batch will require more than one plant

for all caterpillars to develop into adults. Oviposition-site selection is performed in consecutive phases of searching and contact evaluation. *Pieris rapae* butterflies use visual cues as well as olfactory and tactile cues during these phases (Rothschild and Schoonhoven, 1977; Renwick and Radke, 1988). Acceptance of a site may be determined by the balance of positive and negative factors (Renwick and Radke, 1988). Renwick and Radke (1988) suggest that olfaction does not play a role in attraction to a host plant, but may be involved in avoidance of non-host plants. *Pieris rapae* and *P. brassicae* are crucifer specialists and are known to use glucosinolates, toxic secondary metabolites characteristic for Brassicaceae, as oviposition stimulants. Glucobrassicin and sinigrin have been shown to be effective oviposition stimulants for *P. brassicae* and *P. rapae* (Renwick et al., 1992; Van Loon et al., 1992a).

A major signal-transduction pathway involved in induced plant defence is the octadecanoid pathway (Arimura et al., 2005). A central compound in the octadecanoid pathway is jasmonic acid (JA), which has an important role in direct as well as indirect defence against insects in many plant species. In response to JA or MeJA (methyl jasmonate) treatment increased concentrations of several defence compounds have been documented in a range of plant species, e.g., proteinase inhibitors (Moura and Ryan, 2001), polyphenol oxidases (Thaler et al., 1996), nicotine (Baldwin et al., 1996), trypsin inhibitors (Cipollini and Sipe, 2001), glucosinolates (Cipollini and Sipe, 2001; Van Dam et al., 2004; Mewis et al., 2005), as well as increased volatile emission (Boland et al., 1995; Dicke et al., 1999; Koch et al., 1999).

Herbivores are reported to be affected by JA treatment of plants. Several studies have focused on the larval stage of the herbivores, and have shown reduced relative growth rates and leaf consumption (Van Dam et al., 2000; Gols et al., 2003; Van Dam et al., 2004). In field experiments, spraying of JA decreased the abundance of caterpillars, flea beetles, aphids, and thrips (Thaler et al., 2001). Other studies addressed the influence of JA application to plants on the oviposition site selection behaviour of the adult herbivores. These studies showed that JA application can result in induced resistance as well as induced susceptibility (Stanjek et al., 1997; Kessler and Baldwin, 2001; Lu et al., 2004).

Here, we study how JA application affects oviposition of two specialist herbivores on cabbage plants, *Pieris rapae* L. and *P. brassicae* L. (Lepidoptera: Pieridae) that are closely related, yet differ drastically in the amount of eggs they put on one plant. Our study is the first to compare closely related herbivores with a different oviposition strategy, which might affect the consequences of JA-induced responses. JA is known to mediate the induction of chemical defence responses in plants to feeding damage as well as deposition of eggs (Dicke and Van Poecke, 2002; Hilker and Meiners, 2006; Mumm and Hilker, 2006). By using JA we could examine the effects of induced defence responses in cabbage plants. Moreover, JA application has the advantage that visually detectable

damage and the presence of herbivores or eggs are avoided. Finally, JA allows control over the strength of induction through controlled dosages of JA. We hypothesised that JA treatment would inhibit the oviposition of the butterflies. We made solvent extracts to address the identity of the active plant compounds that influenced butterfly behaviour. Rather than testing whole-leaf extracts, we extracted the glucosinolates from the surface of both control and JA-treated plants and tested the oviposition preference of the butterflies for these glucosinolate fractions on a neutral substrate. Furthermore, we included a control experiment to exclude a potential direct effect of JA on oviposition behaviour. We address the following questions: (1) does JA-treatment of cabbage plants affect host plant selection of the two *Pieris* butterfly species, (2) are there differences between solitary and gregarious butterflies, (3) does JA-treatment affect glucosinolate levels in leaf surface extracts, and (4) do changes in glucosinolate levels determine the changes in oviposition preference?

Methods and materials

Plants and insects

Brussels sprouts plants, *Brassica oleracea* var. *gemmifera* L. (Brassicaceae) cultivar Cyrus were grown from seed in a greenhouse in plastic pots (11 x 11 x 11 cm) at 20-28 °C, 40-80 % RH and a 16L:8D photoperiod. All experiments were conducted with 6-7 wk old plants. Stock colonies of the large cabbage white *P. brassicae* and the small cabbage white *P. rapae* were maintained on Brussels sprouts plants in a climatised room at 20-22 °C, 50-70 % RH and a 16L:8D photoperiod.

Chemical analysis

Brussels sprouts plants were sprayed with 0.1 mM JA or control solution. JA (+/- jasmonic acid, purity > 97 %; Sigma-Aldrich, St Louis, USA) was applied to the surface of the leaves, i.e., plants were sprayed with a JA-solution with 0.1 % Tween 20 until run-off or just with 0.1 % Tween 20 for the control treatment. The next day, glucosinolates (GLS) were extracted from the surface of the intact Brussels sprouts leaves. Each sample consisted of 4 leaves (between the 3rd to 6th leaf from the base of a plant) that were cut at the base of the petiole. Directly after cutting, the lamina was dipped for 5 sec in 300 ml of dichloromethane, and after a 5 sec interval they were dipped for 5 sec in 150 ml of methanol (Städler and Roessingh, 1990; Van Loon et al., 1992a; Griffiths et al., 2001). The methanol was evaporated from the crude methanol dip-volume with a rotary evaporator (IKA-Werke GmbH, Staufen, Germany). For each treatment 11 plants were sampled. The extract was redissolved in methanol, desulphatased on a DEAE-Sephadex A25 column, and separated on a reverse phase C-18 column using HPLC as described in Van Dam et al. (2004). Glucosinolate detection was performed with a PDA detector (200 – 350 nm) with 229 nm as the integration wavelength. Sinigrin (sinigrin monohydrate, ACROS, New Jersey, USA) was used as an external standard. We used the correction factors at 229 nm from

Buchner (1987) and the EC (EC, 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). The surface area was measured directly after dipping and the dry mass of the leaves was measured after drying at 50 °C for 72 hr. The GLS content was calculated in pmol per cm^2 leaf material.

Herbivore oviposition preference test

Pieris adults emerged from pupae in a large oviposition cage (67 cm x 100 cm x 75 cm) in a greenhouse compartment at 22-24 °C and 50-70 % RH. Apart from natural daylight, cages were illuminated by sodium vapour lamps (type SON-T, 500 W, Philips, The Netherlands) from 8:00 a.m. till 2:00 p.m.. In this cage they were provided with a 10 % sucrose solution and an oviposition substrate, depending on the experiment a plant or an artificial leaf made of green cardboard paper sprayed with sinigrin. For the experiments, one male and one female butterfly were introduced per oviposition cage (67 cm x 50 cm x 75 cm) in the same greenhouse compartment, on the day before the experiment. In these cages the butterflies were also provided with sucrose solution. At 8:30 a.m., the treated leaves or papers and respective controls were introduced in the cages, and the butterflies were allowed to oviposit until the beginning of the afternoon. At 2:00 p.m., the leaves were removed and the number of eggs counted. The experiments were carried out in several cages per day and 3-4 days per treatment with new pairs of butterflies each day, adding up to a total of 24-36 independent replicates.

Surface application of JA

The effect of JA-induced changes in Brussels sprouts plants on butterfly behaviour was tested in oviposition experiments with *P. brassicae* and *P. rapae*. Three concentrations of JA solution, 0.01 mM, 0.1 mM, and 1 mM, corresponding to approximately 1.25 µg, 12.5 µg, and 125 µg JA/g fresh weight (or 0.25 nmol, 2.5 nmol, and 25 nmol JA/cm^2) respectively, were sprayed on the plants and tested against a control (plants treated with 0.1 % Tween 20). The next morning, just before the start of the experiment the 4^{th}, 5^{th}, and 6^{th} leaves from the base of the plants were cut, and their petioles were placed directly in a vial with tap water and introduced into the cages with butterflies.

Systemic uptake of JA

For *P. rapae* two application methods were used to assess the effect of JA-induced changes in Brussels sprouts plants on oviposition preference. For the second application method, the 4^{th}, 5^{th}, and 6^{th} leaves were cut from untreated plants and placed in a 0.1 mM aqueous JA solution 22 hr before start of the experiment. Total uptake of the solution was on average 6.3 ± 1.5 ml per control leaf and 6.0 ± 1.6 ml for JA-treated leaves (corresponding to approximately 20 µg JA/g fresh weight or 5

nmol JA/cm^2, assuming homogeneous distribution over the leaf tissue after uptake).

Effect of pure JA on oviposition preference

In the next experiment, green cardboard paper sprayed with an oviposition stimulant was used to test the effect of pure JA on the oviposition behaviour of *P. rapae* on an inert substrate. Sinigrin has been shown to be a suitable oviposition stimulant for *Pieris* butterflies (Van Loon et al., 1992a) and was therefore used to stimulate oviposition on the artificial substrate in this experiment. The paper (8 x 11.5 cm) was treated with 1 ml of a 5 mM sinigrin solution (Janssen Pharmaceutica, Tilburg, The Netherlands) by spraying it with a Desaga chromatographic sprayer (Heidelberg, Germany). Subsequently, after drying, papers were sprayed with either 1 ml of a 1 mM JA solution or water (control substrates) just before the test (210 µg JA/carton or 11 nmol JA/cm^2).

Bioassays with purified glucosinolate (GLS) fractions

GLS were extracted from the leaf surface as described for the chemical analysis. For each treatment, control and 0.1 mM JA, 60 plants were used for the extraction, of which 4 - 5 leaves per plant were dipped. Subsequently, the extracts were fractionated following the protocol of Sørensen (Sørensen, 1990). The GLS fractions were stored in the freezer until analysis. The GLS were dissolved in methanol to make two concentrations, one corresponding to the amount of GLS extracted from the material of two plants in 0.8 ml and one concentration corresponding to the amount of GLS from one leaf in 0.8 ml. Hereafter, we will express these concentrations in gram leaf equivalents (gle). One gle corresponds to the amount of GLS extracted from 1 g fresh and intact leaf. With an average weight of 6 g per leaf the highest concentration corresponds to 48 gle and the lower concentration to 6 gle. With a sprayer, a volume of 0.8 ml of one of the solutions was sprayed on green paper following the same method as described above for the test of pure JA. *Pieris rapae* butterflies were offered a two-choice situation, with one paper sprayed with GLS extracted from control plants, and one paper with GLS from JA-treated plants.

Performance of *Pieris rapae* caterpillars

The development of first instar caterpillars to pupae was observed on control and JA-treated plants. Control plants were sprayed with a 0.1 % Tween 20 solution, and JA-treated plants with a solution of 0.5 mM JA with 0.1 % Tween 20. Thirty newly hatched *P. rapae* caterpillars were evenly distributed over two plants per treatment, 24 hr after treatment, and placed in cages (67 cm x 50 cm x 75 cm) in a greenhouse compartment at 22-24 °C and 50-70 % RH. The plants were replaced with new plants twice a week, so that the maximum time between induction and larval feeding never exceeded 5 d. The number of days until pupation and pupal weight was recorded.

Statistical analyses

Each individual butterfly female was subjected to a two-choice situation, in which most individuals oviposited on both control and JA-treated leaves. Since the egg load differed between individuals, the number of eggs on each treatment per individual was treated as a paired sample. The oviposition data for *P. rapae* were normally distributed; therefore they were analysed with a paired *t*-test. The oviposition data for *P. brassicae* were not normally distributed and therefore analysed with the non-parametric equivalent of the paired *t*-test, the Wilcoxon matched-pair signed-ranks test. The data on the developmental time of the caterpillars in the performance test were not normally distributed and analysed with a Mann-Whitney *U* test for differences between the treatments. Pupal weight was normally distributed and analysed with an ANOVA. Changes in GLS content were analysed with a Mann-Whitney *U* test. Statistical analyses were performed with SPSS 11.0.

Results

Chemical analysis

Five GLS were detected in *B. oleracea* leaf surface samples: glucoiberin, sinigrin, 4-hydroxyglucobrassicin, glucobrassicin, and 4-methoxyglucobrassicin (Table 1). No significant difference was detected between JA-treated and control leaves for the total amount of GLS per cm^2. The amounts of glucobrassicin, the most abundant glucosinolate in these samples, 4-methoxyglucobrassicin and sinigrin, did not significantly differ between control and JA-treated leaves. The amounts of glucoiberin and 4-hydroxyglucobrassicin collected in the leaf surface extracts were significantly lower for JA-treated leaves compared to control leaves (Table 1). The same results were obtained when calculated for the GLS content expressed as nmol per mg dry weight (not shown).

Table 1. Glucosinolate content in surface extracts of *Brassica oleracea* leaves in pmol/cm^2 for control and JA-treated plants (* P<0.05)

Compound	Control treatment Median (range)[a,b]	JA-treatment Median (range)[a,c]	Z	P
Glucoiberin	10.7 (6.3 - 21.7)	0 (0 - 6.0)	-2.713	0.007 *
Sinigrin	9.3 (0 - 23.5)	4.2 (0 - 6.8)	-1.774	0.076
4-Hydroxyglucobrassicin	0.7 (0 - 6.1)	0 (0 - 0)	-2.207	0.027 *
Glucobrassicin	34.9 (18.4 - 98.2)	88.6 (38.6 - 132.5)	-1.479	0.139
4-Methoxyglucobrassicin	0.5 (0 - 2.5)	0 (0 - 1.3)	-1.370	0.171
Total amount of glucosinolates	64.1 (27.0 - 155.7)	90.0 (42.9 - 138.8)	-0.563	0.573

[a] interquartile range from 1st to 3rd quartile
[b] $N = 10$
[c] $N = 11$

Herbivore oviposition preference: Surface application of JA

Since *P. brassicae* lays its eggs in batches, both the number of egg batches and the number of eggs per leaf were counted. For the 1 mM JA treatment,

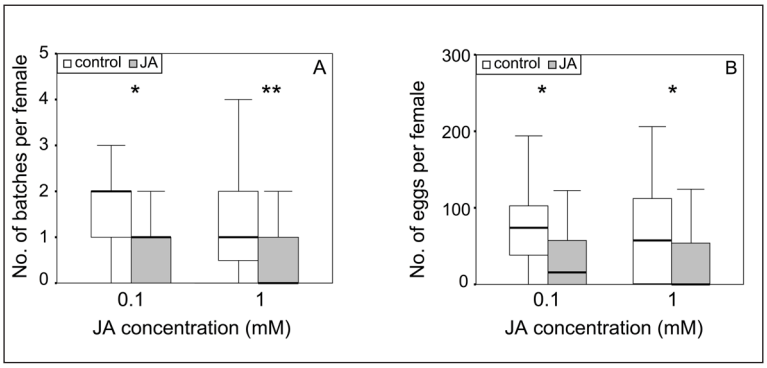

Figure 1. *Pieris brassicae* oviposition on control and JA-treated plants. Two concentrations of JA were tested against a control. Median and quartiles are given, asterisks indicate statistical differences between the preference for control and JA-treated plants (* = P <0.05, ** = P < 0.01, Wilcoxon matched-pair signed-ranks test). (A) Egg batches per female per leaf. (B) Eggs per female per leaf.

the number of batches was significantly lower on JA-treated leaves than on control leaves (N = 36, Z = -2.628, P = 0.009, Wilcoxon matched-pair signed-ranks test), and the total number of eggs was significantly lower as well (N = 36, Z = -2.035, P = 0.042, Wilcoxon matched-pair signed-ranks test). For the 0.1 mM JA treatment, the result was similar (N = 27, batches: Z = -2.223, P = 0.026; eggs: Z = -2.138, P = 0.032, Wilcoxon matched-pair signed-ranks test) (Figure 1). Experiments with 0.01 mM JA application did not show discrimination by the butterflies between the treated and control leaves (results not shown). Also *P. rapae* butterflies significantly preferred to oviposit on control leaves compared to JA-treated leaves (Figure 2). The leaves treated with the two highest concentrations of JA tested, 1 mM and 0.1 mM, were significantly avoided in favour of the control leaves (paired t-test, respectively t = 3.805, df = 23, P = 0.001 and t = 3.681, df = 23, P = 0.001). The lowest concentration of JA tested, i.e., 0.01 mM, did not affect the distribution of eggs over the leaves (t = -0.662, df = 23, P = 0.52, paired t-test).

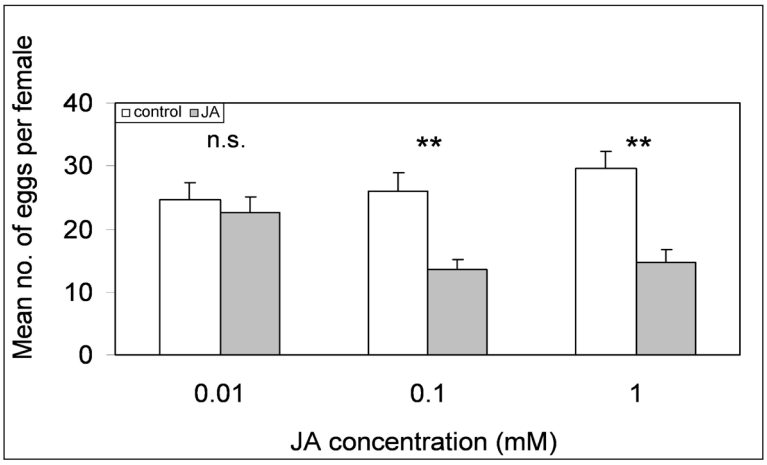

Figure 2. *Pieris rapae* oviposition preference (measured as the number of eggs per female per leaf) between control and JA-treated *B. oleracea*. Three concentrations of JA were tested against a control in 24 replicated experiments for each concentration. Mean numbers of eggs per female + SEM are given, asterisks indicate statistical differences between the preference for control and JA-treated plants (n.s. P > 0.05, ** P < 0.01, paired t-test).

Systemic uptake of JA

In the experiment with *P. rapae* that employed systemic uptake of JA through the petiole, the same result was obtained: the number of eggs on the JA-treated leaves (10.0 ± 1.57) was lower than on the control (19.12

± 2.82) leaves (t = 3.976, df = 31, P < 0.001, paired t-test)

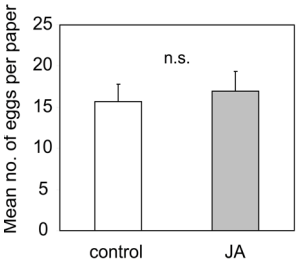

Figure 3. Oviposition of *P. rapae* on green paper sprayed with sinigrin plus JA and green paper with sinigrin only. Mean number of eggs per female per leaf + SEM, asterisks indicate statistical differences between the preference for control and JA-treated plants (n.s. = P > 0.05, paired t-test, N = 27).

Effect of pure JA on oviposition preference

When paper treated with sinigrin and JA was compared to paper with only sinigrin, there was no difference in the number of eggs the butterflies deposited on the two substrates (t = -0.438, df = 26, P = 0.67, paired t-test) (Figure 3). These results show that the observed effect of the JA-treatment on herbivore oviposition behaviour was due to induced changes in leaf tissue rather than to a direct repellent or deterrent effect of JA itself.

Bioassays with purified glucosinolate fractions

The butterflies did not discriminate between the two GLS-fractions (Table 2), the number of eggs on paper with the GLS from control plants and the number of eggs on paper with GLS from JA-treated plants was not different for both concentrations (concentration 6 gle: Z = -1.514, N = 20, P = 0.130, Wilcoxon matched-pair signed-ranks test; 48 gle: t = -0.523, df = 19, P = 0.607, paired t-test) (Figure 4).

Table 2. Glucosinolate (GLS) content in fractions from leaf surface extracts from control and JA-treated plants in pmol/cm², Z and P-values of Mann-Whitney U test

Glucosinolate	GLS from control plant	GLS from JA-treated plant
Glucoiberin	4.9	3.9
Sinigrin	3.1	5.1
4-Hydroxyglucobrassicin	not detected	not detected
Glucobrassicin	16.0	81.0
4-Methoxyglucobrassicin	not detected	0.8

Performance of *Pieris rapae* caterpillars

About two third of the caterpillars survived until pupation, and a similar number of caterpillars reached the pupal stage on both treatments, 19 on control and 18 on JA-treated plants (Figure 5). The caterpillars on JA-treated plants pupated on average after 15 d, while the caterpillars on the control plants pupated significantly sooner, on average after 13 d (Mann-Whitney U, Z = -4.071, P < 0.001). The average pupal weight on the control plants, 165 ± 3.4 mg, was similar to that on the JA-treated plants, 158 ± 3.3 mg (ANOVA, F = 2.665, df = 1, P = 0.112).

Discussion

Our data show that JA-treatment of Brussels sprouts leaves reduces the acceptance of the leaves for oviposition by *Pieris rapae* and *P. brassicae* in a similar way. Treatment of the leaves with 0.1 or 1 mM JA reduced the proportion of eggs the butterflies laid on these leaves. A concentration of 0.01 mM JA did not change the oviposition preference of the butterflies. The former concentrations are comparable to the concentrations of JA or MeJA that were applied to several plant

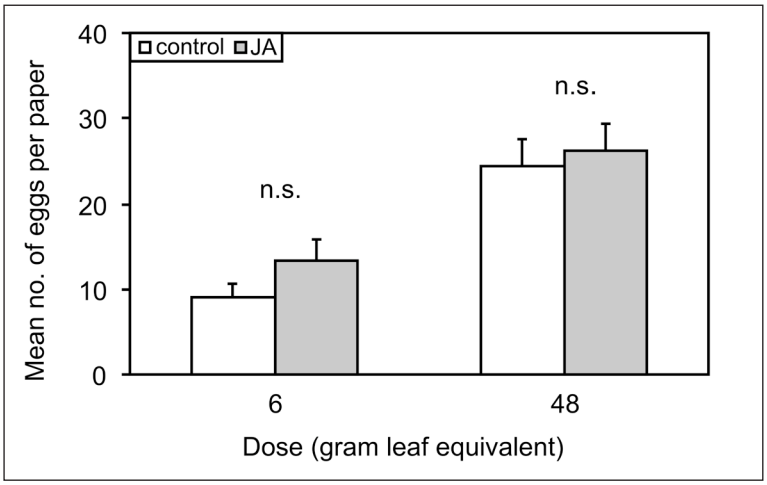

Figure 4 (left). Oviposition of *P. rapae* on green paper sprayed with purified GLS-fractions from leaf surface extracts of control (white bars) and JA-treated (grey bars) plants. Mean number of eggs per female per leaf + SEM, asterisks indicate statistical differences between the preference for control and JA-treated plants (n.s. = P > 0.05, paired t-test, N = 20).

species in other studies and reduced development of *Spodoptera exigua*, *Trichoplusia ni*, *Manduca sexta*, thrips, and aphids (Thaler et al., 1996; Avdiushko et al., 1997; Van Dam et al., 2000; Omer et al., 2001), and abundance of *M. quinquemaculata*, *S. exigua*, thrips, and flea beetles in the field (Kessler and Baldwin, 2001; Thaler et al., 2001). For cabbage plants, Lu et al. (2004) found inducible resistance in a susceptible *Brassica* species (Chinese cabbage, *B. campestris* L.) and induced susceptibility in a resistant *Brassica* species (common cabbage, *B. oleracea*) for *Plutella xylostella* L..

To exclude that JA itself caused the above effect, we tested the phytohormone on an inert substrate and studied two different application methods to the leaf material. We considered this an essential control which is lacking in other studies. The results of these experiments show that it was not JA itself that caused the difference in oviposition preference of the *Pieris* butterflies between the control and JA-treated leaves, thus providing proof that processes in the plant induced by the JA-treatment changed the acceptability of the leaves. Also for MeJA it has been reported that development of cabbage looper or tobacco hornworm larvae was not affected when MeJA was added to an artificial diet, but it was retarded when MeJA was applied to cabbage or tobacco plants (Avdiushko et al., 1997).

In leaf surface extracts of JA-treated and untreated Brussels sprouts plants we found five glucosinolates. After JA application, glucobrassicin, the major glucosinolate in the *B. oleracea* cultivar we used, occurred at a level twice as high as in control plants, and glucoiberin and 4-hydroxyglucobrassicin concentrations decreased after JA-treatment. The total glucosinolate content did not change significantly. However, most other studies on glucosinolate content in Brassicaceous plants after induction by JA- or MeJA-treatment or insect attack reported an increase

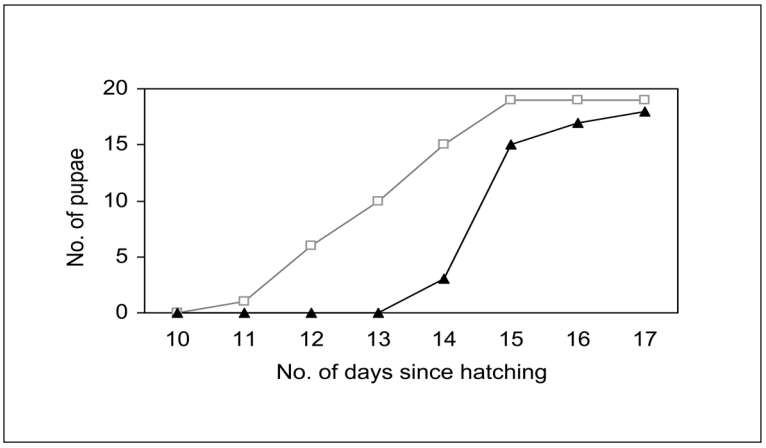

Figure 5 (right). Development time of *P. rapae* caterpillars from hatching until pupation on control (□) and JA-treated (▲) plants. Cumulative number of pupae per treatment.

in glucosinolates, although there is substantial variation among different plant species, or even genotypes, and type of induction (Bodnaryk, 1994; Cipollini and Sipe, 2001; Mikkelsen et al., 2003; Mewis et al., 2005). Moreover, glucosinolates may not be evenly distributed throughout the leaf. We measured glucosinolate content in a surface extract after 24 hr, whereas most studies measured glucosinolate content in whole leaf extracts, and after a longer induction time. Recently, Reifenrath et al. (2005) postulated that the wax layer does not contain glucosinolates, and the polar glucosinolates that are found using the solvent extraction method are washed from the inner leaf to the outside through the stomata. Nevertheless, we chose a surface extraction method because the butterflies retrieve chemosensory information from the surface of the leaf, since they do not damage the leaf before ovipositing. Surface extracts are therefore likely to give a better reflection of the chemosensory information used than whole leaf extracts.

Both butterfly species distinguish between induced and non-induced leaves, most likely based on chemical differences, as JA-induced leaves do not display herbivore presence or damage. The different levels of two out of five glucosinolates in the leaf surface extracts may provide a chemosensory basis for the oviposition preference observed, although the isolated GLS from the two treatments yielded no differences in acceptance of the paper for oviposition. While the isolated GLS on paper stimulated oviposition behaviour, they appear not to be the main cue to discriminate between the JA-induced and non-induced cabbage plants.

We did not quantify other chemicals, stimulants, deterrents, or precursors, that might mediate preference behaviour, like isothiocyanates, terpenoids, other glycosides, or amino acids (Huang et al., 1993; Renwick and Chew, 1994; Soldaat et al., 1996; Agrawal and Kurashige, 2003). Both *P. rapae* and *P. brassicae* can perceive a broad range of chemicals (Van Loon et al., 1992b; Hern et al., 1996). Electroantennogram responses to a range of plant volatiles were similar for both species (Van Loon et al., 1992b), although host plant selection by *Pieris* butterflies appears largely

based on contact chemoreception rather than on olfaction (Renwick & Chew, 1994). Host plant selection is suggested to depend on a balance of stimulants and deterrents and not just on the detection of presence or absence of particular compounds (Huang et al., 1993; Bruce et al., 2005). Therefore, glucosinolates in combination with other stimulants or deterrents may determine the acceptance of a host plant by the butterflies.

The octadecanoid pathway, in which JA is a key molecule, is involved in induction of synomones in response to oviposition as well as to herbivore damage (Meiners and Hilker, 2000; Dicke and Van Poecke, 2002; Hilker and Meiners, 2006; Mumm and Hilker, 2006). JA-treatment of plants has been shown to result in emission of synomones that attract natural enemies like predatory mites and parasitoids (Dicke et al., 1999; Hilker and Meiners, 2002; Van Poecke and Dicke, 2002; Hilker and Meiners, 2006; Chapter 4 and 5). This attraction will result in a higher natural enemy density around damaged plants, and therefore it is advantageous for the herbivores to avoid oviposition on induced plants. Moreover, intact plants lack competitors, either intra- or interspecific. Herbivores may use induced plant cues to detect the presence or absence of other herbivores on a plant, especially because plant cues are, although less reliable, often easier to detect than cues from the herbivores themselves (Vet and Dicke, 1992).

Furthermore, the induced plants may affect the herbivores directly by influencing the performance of their offspring. For *P. rapae*, the development time differed between caterpillars feeding on JA-induced and caterpillars feeding on non-induced plants. The development of the caterpillars to pupae took longer on the induced plants, which exposes them to natural enemies for a longer time and gives them a disadvantage in the competition for resources with other herbivores. These results comply with those of Agrawal and Kurashige (2003), who showed that growth of *P. rapae* larvae was reduced on herbivore-induced Brassicaceae.

In summary: (1) JA-treatment of *B. oleracea* results in avoidance of host plants by the two *Pieris* butterflies, (2) the related gregarious and solitary butterfly species tested here responded in a similar fashion to JA-treated plants, (3) JA-treatment reduced the contents of 2 out of 5 glucosinolates in leaf surface extracts of Brussels sprouts plants, and (4) the purified GLS fractions could not explain the observed avoidance behaviour. These results indicate that JA-induced infochemicals play an important role in host plant selection behaviour of these butterflies; however, the phytochemicals involved still have to be elucidated.

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TIBOR BUKOVINSZKY

Chapter 4

Jasmonic acid-induced changes in *Brassica oleracea* attract parasitoids: effects of time and dose and differences with induction by herbivores

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Joop J.A. van Loon & Marcel Dicke

Abstract

Feeding by biting–chewing herbivores induces the plant hormone jasmonic acid (JA), a central compound in the octadecanoid plant defence signaling pathway. In this study the defence response of Brussels sprouts plants was chemically induced by exogenous application of JA. We studied the effect of this induction on volatile emission of the plant and on host-location behaviour of parasitoid wasps. Three species of parasitoid wasps, *Cotesia glomerata*, *C. rubecula* and *Diadegma semiclausum*, differing in host range and host specificity, were tested for their behavioural responses to herbivore-induced, JA-induced and non-induced plants. All three species responded in a similar fashion; they were attracted to JA-induced plants compared to control plants; however, they preferred herbivore-induced plants over JA-induced plants. JA-induced plants produced larger quantities of volatiles than herbivore-induced and control plants; this implies that not quantity, but quality of the volatile blend is most important in the host-location behaviour of the wasps. *Cotesia glomerata* was attracted to the plants within a few hours after JA-induction, and the plants remained attractive to the parasitoids for more than five days, while herbivore-induced plants did not attract parasitoids five days after removal of the herbivores. Application of doses ranging from 10 μM to 1 mM JA resulted in attraction of *C. glomerata* to the treated plants, a lower dose of 1 μM JA was not effective. Therefore we conclude that JA-application has the advantage of quantitatively controlling the strength of induction, but does not fully mimic actual herbivore feeding in terms of parasitoid response and volatile emission.

Introduction

In response to damage, the phenotype of an individual plant changes because of the induction of defences. This may affect the insect community around the plant; either directly by affecting the herbivores and their natural enemies or indirectly by modifying the interactions between the different trophic levels (Price et al., 1980; Van Zandt and Agrawal, 2004; Schoonhoven et al., 2005; Takabayashi et al., 2006). We focused our studies on the effects of volatiles that plants release in response to herbivory on the third trophic level. Herbivore-induced plant volatiles have been shown to attract natural enemies of the herbivores attacking the plant (Turlings et al., 1990; Steinberg et al., 1992; Geervliet et al., 1994) and the same infochemicals can affect herbivore behaviour as well (Dicke and Vet, 1999; Sabelis et al., 1999). The types and amounts of volatiles emitted by the plants differ depending on plant species, attacking herbivore species, herbivore developmental stage and abiotic factors (Turlings et al., 1993; Takabayashi et al., 1994; Takabayashi and Dicke, 1996; Dicke and Van Poecke, 2002; Gouinguené and Turlings, 2002; Hilker and Meiners, 2002).

Herbivore-induced plant volatiles play an important role in parasitoid host-location behaviour. As their hosts generally are just minute components of the environment, and under selection pressure to escape parasitism and predation, cues produced by hosts are generally difficult to detect. However, host cues are the most reliable cues in indicating the presence of a host. In view of the higher amounts released, plant volatiles are usually easier to detect, but supposedly with lower reliability regarding host presence (Vet and Dicke, 1992). However, when herbivore-induced plant volatile emission is specific for the attacker, and differs depending on the herbivore species damaging the plant, as well as on the developmental stage of the herbivore, it can provide both reliable and detectable information for parasitoids (Vet et al., 1991). For the model plant species in this study, Brussels sprouts, it is known that caterpillar-infested plants are attractive to parasitoids such as *Diadegma semiclausum* and several *Cotesia* spp. (Blaakmeer et al., 1994a; Geervliet et al., 1996; Ohara et al., 2003). Already within one hour after herbivore infestation females of the parasitoid wasp *Cotesia glomerata* are attracted to Brussels sprouts plants (Scascighini et al., 2005).

The volatile blends emitted by plants can be manipulated by interfering with the signal transduction pathways leading to volatile emission. Manipulation of the volatile emission of a plant using an elicitor allows the investigation of the possible effects of plant volatiles on community ecology. The use of an elicitor has the advantage of being able to induce (part of) the volatile blend without removal of plant tissue and offers the possibility to apply a controlled dosage, whereas it is difficult to control the amount of damage inflicted by herbivore feeding. In this study the phytohormone jasmonic acid (JA) is used to manipulate the volatile emission of Brussels sprouts plants. JA is an important signalling

compound in pathways regulating induced direct as well as indirect defence to herbivorous arthropods (Dicke and Van Poecke, 2002). JA or MeJA (methyl jasmonate) treatment has been reported to induce volatile emission, similar to herbivore induction, extrafloral nectar production, increased levels of endogenous secondary metabolites, reduced development and oviposition of herbivores and increased attraction of predators and parasitoids and enhanced parasitism rates in a wide variety of plant species, e.g. Lima bean (Dicke et al., 1999; Gols et al., 2003; Heil, 2004), tomato (Thaler, 1999a; Thaler et al., 2001), tobacco (Avdiushko et al., 1997; Kessler and Baldwin, 2001), cotton (Omer et al., 2001; Rodriguez-Saona et al., 2001), maize (Hopke et al., 1994; Ozawa et al., 2004), rice (Lou et al., 2005), and field elm (Meiners and Hilker, 2000). Jasmonates have also been used successfully for induction of several Brassicaceous species such as *Arabidopsis thaliana* (Van Poecke and Dicke, 2002; Mewis et al., 2005), common cabbage (Ibrahim et al., 2005), and oilseed rape (Loivamaki et al., 2004). This wide range of species illustrates that this phytohormone is ubiquitous in plant-insect interactions and we hypothesised it to be involved in the defence of Brussels sprouts plants against herbivorous insects as well.

To gain insight in the effects of modified volatile production on interactions with parasitoid wasps, we investigated the effects of JA-treatment on the volatile release of Brussels sprouts, *Brassica oleracea* var *gemmifera,* and on the behaviour of several associated parasitoid wasps. We selected three species of parasitoids, *Cotesia rubecula* (Marshall), *Costesia glomerata* L. (Hymenoptera: Braconidae) and *Diadegma semiclausum* (Hellén) (Hymenoptera: Ichneumonidae), that all parasitise caterpillars on Brassicaceous plants, but differ in their host range and specificity. While both *Cotesia* wasps use *Pieris* caterpillars as their hosts, *D. semiclausum* is a specialist parasitoid of *Plutella xylostella*. *Cotesia glomerata* accepts *Pieris brassicae*, *P. rapae* and *P. napi*, whereas *C. rubecula* is more specialised in its choice of hosts for oviposition, and prefers *P. rapae* as a host, only rarely accepting other hosts (Brodeur et al., 1996). We studied whether parasitoids that differ in host range and specificity also differ in their response to JA-induced plants. Subsequently, the effect of JA-induction on parasitoid behaviour was studied in more detail; *C. glomerata* attraction to plants was tested at several time points after induction and in response to treatment with different concentrations of JA. Finally, we analysed the volatile blends emitted by non-induced, herbivore-damaged and JA-treated plants to compare the relative effects of herbivory and JA.

Materials and methods

Plant material

Brussels sprouts, *Brassica oleracea* L. var. *gemmifera* cultivar Cyrus (Brassicaceae) were grown from seed in a greenhouse in plastic pots

(11 × 11 cm) at 20–28 °C, 40–80 % RH and a 16L:8D photoperiod. All experiments were conducted with 6–7 week old plants.

Insects

Stock colonies of the large cabbage white butterfly *Pieris brassicae* L., the small cabbage white *P. rapae* L. (Lepidoptera: Pieridae) and the diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) were maintained on Brussels sprouts plants in a climatised room at 20–22°C, 50–70% RH and a 16L:8D photoperiod. The parasitoid wasps *Cotesia rubecula* and *C. glomerata* were reared in a greenhouse at 22–24°C, 50–70% RH and 16L:8D photoperiod. *Cotesia rubecula* and *C. glomerata* were maintained on *P. rapae* and *P. brassicae* respectively, both feeding on Brussels sprouts plants. *Diadegma semiclausum* was reared on *Pl. xylostella* feeding on Brussels sprouts plants in a climate room at 20–22°C, 50–70% RH under a 16L:8D photoperiod. All adult wasps emerged in a cage without any plants or hosts, were fed with honey and kept under the same climate conditions as under which they were reared until use in the experiments.

Parasitoid preference

Behavioural experiments with parasitoid wasps were conducted to compare the attractiveness of plants subjected to different induction treatments for the three wasp species. JA-treated plants were tested in dual-choice experiments against control plants and against caterpillar-infested plants.

Plant treatments

For the JA-treatment in the first experiment, the plants were sprayed with 20 ml of a 1 mM JA (+/- jasmonic acid Sigma-Aldrich, purity > 97%) aqueous solution containing 0.1 % Tween 20 as surfactant, which corresponds to approximately 140 µg JA/g leaf fresh weight, and the control plants with a 0.1% Tween 20 solution (Van Poecke and Dicke, 2002). The caterpillar treatment consisted of plants infested with herbivores: 12 second instar *Pl. xylostella* for *D. semiclausum* choice experiments (comparable to densities observed in cabbage plants in the field) and 30 first-to-second instar *P. rapae* for both *Cotesia* spp (based on experiments with Brussels sprouts plants and JA by Z. Szendrei & M. Dicke, unpublished results). Caterpillars were introduced on the plants 24 ± 2 hr before the experiments.

Preference behaviour of three parasitoid wasp species – windtunnel

The behaviour of the three parasitoid wasp species was tested in a windtunnel (as described in detail by Geervliet et al., 1994). The windtunnel conditions were set at 26 ± 2 °C, 60–70% RH, a light intensity of 24 ± 2 µmol $m^{-2}s^{-1}$ (Quantum meter QMSW-SS, Apogee instruments Inc., Logan, UT, USA) and a wind speed of 20 cm/s (Thermisches anemometer, Wilh. Lambrecht GmbH, Göttingen, Germany). The adult wasps were provided with water and honey, but had no experience with plants or caterpillars until the experiment. Female wasps were separated

from male wasps on the day before the experiment. Wasps of 3–6 days old were used for all experiments and were assumed to have mated. The female parasitoid wasps were released at approximately 60 cm distance downwind from the two plants. The parasitoids were individually released on a small piece of leaf damaged by their respective caterpillar host, from which caterpillars and faeces had been removed. After release in the windtunnel, an individual parasitoid female was observed until it landed on one of the plants (choice) or for a maximum of 10 minutes without landing, after which it was recorded as not having made a choice (no choice). Wasps that did not make a choice were discarded from the analysis. Each wasp was used only once. Position of plants that had been exposed to different treatments was alternated after a maximum of five wasps to exclude possible directional bias. All experiments, control vs. JA and JA vs. herbivore-infested, for all three species, were tested on at least five days, with new sets of plants.

Preference behaviour of Diadegma semiclausum – without airflow

To test the importance of an air flow in parasitoid orientation behaviour we tested *D. semiclausum* attraction to the same treatments in another experimental set-up. This set-up consisted of a glass cage (97 cm × 115 cm × 95 cm) without any air flow in which two plants were placed 60 cm apart. The wasps were released in the middle of the cage, half way between the two plants. The abiotic conditions were similar to the windtunnel experiments and we performed the same dual-choice tests. Similar to the windtunnel experiments the wasps were released on a small piece of leaf previously damaged by *Pl. xylostella*.

Dose-effect relationship

After testing one concentration (1 mM) on different parasitoid species, we tested the response of one parasitoid, *C. glomerata*, to plants treated with different concentrations of JA. A series of 1 µM, 10 µM, 100 µM and 1 mM JA was tested against control plants in the windtunnel; each plant was sprayed until run-off. Several concentrations were tested on the same experimental day and each concentration was tested on at least five different days. The effect on parasitoid behaviour was investigated in the windtunnel described above.

Effect of time since treatment

To test when plants become attractive to the parasitoids after JA treatment and how long this attraction lasts, the time of testing after induction with 1 mM JA was varied from 1 to 120 hours. In the windtunnel we investigated the response of *C. glomerata* to plants that were treated 1, 2, 3, 6, 24, 48, 72 or 120 hours before the test with JA, against a control plant (treated with a Tween 20 solution at the same time). We used new plants at each time point and tested parasitoid responses on at least five different days per JA dose. The set-up was the same as described above. In order to compare induction with JA to induction by herbivores, we infested plants with 5 or 15 *P. rapae* first-instar larvae and let them feed

for 24 hours. Subsequently, we removed all caterpillars and tested the plants against intact plants 48 and 120 hours after removal.

Attraction to an inert substrate treated with JA

To rule out that the observed effect of JA-treatment is due to JA itself, rather than the result of induction of plant volatiles, we tested the response of *C. glomerata* to an inert substrate treated with JA. Just prior to the experiment 1 ml of 5 mM JA was sprayed onto green paper (8 x 11.5 cm) with a Desaga chromatographic sprayer (Heidelberg, Germany), which amounts to a concentration of approximately 10 µg JA/cm^2 (roughly corresponding to the amount of JA that would be sprayed on the plant per cm^2 at a dose of 2 mM). In the windtunnel a control substrate (green paper sprayed with water) and JA-treated substrate were tested against each other; next to each substrate an excised leaf (excised from an intact Brussels sprouts plant) was placed. Landings on both leaf and substrate were recorded.

Volatile analysis

For the chemical analysis of the volatiles emitted by plants subjected to different treatments, plants were treated the same way as for the parasitoid preference experiments. The headspace collection was performed in a climate room at 22-24°C, 50-70% RH and a light intensity of 95 ± 5 µmol $m^{-2}s^{-1}$ (Quantum meter QMSW-SS, Apogee instruments inc., Logan, UT, USA). Pressurised air was filtered over silica gel, a molecular sieve (4Å) and activated charcoal, and led through a 30 l clean glass jar. Overnight, clean air was led through the jar at a flow rate of 100 ml/min to remove any remaining volatile contaminants. Just before placing the plant in the jar, the pot of the plant was removed and the roots and soil were packed tightly in aluminium foil. The plant was placed in the jar, which was closed with a glass lid with a Viton® O-ring in between and the lid was tightly closed with a metal clamp. First the jar with the plant was purged for 1 hour with an air flow through the jar of 50 ml/min. Subsequently, headspace volatiles were collected at the outlet of the jar on a glass tube filled with 90 mg Tenax-TA 25/30 mesh for 4 hours at a flow rate of 40 ml/min. After collection, the tube was closed and stored at room temperature until GC-MS analysis. Two plants of different treatments were sampled at the same time, and five replicates per treatment were sampled and analysed. Headspace samples were analysed with a Varian 3400 GC connected to a Finnigan MAT 95 MS. The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack) at 250° C for 10 min and flushing with helium at 14 ml/min. The released compounds were cryofocused in a cold trap (0.52 mm ID deactivated fused silica) at a temperature of -85°C. By ballistic heating of the cold trap to 220°C the volatiles were transferred to the analytical column (DB-5ms J&W, Folsom, CA, 60 m x 0.25 mm ID, 0.25 µm - film thickness). The temperature program started at 40°C (4-min hold) and rose 5°C min^{-1} to 280°C (4-min hold). The column effluent was ionised by electron impact (EI) ionisation at

70 eV. Mass scanning was done from 24 to 300 *m*/*z* with a scan time of 0.7 s/d and an interscan delay of 0.2 s. Compounds were identified by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index.

Statistical analysis

The parasitoid choices between two odour sources were statistically analysed using the binomial test. Differences among the percentages of wasps making a choice were tested using a contingency table test on the absolute numbers (SAS 9.1). The volatile patterns of differently treated plants were analysed using Principal Component Analysis (PCA) and Projection to Latent Structures-Discriminant Analysis (PLS-DA) using the software program SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). PCA obtains so-called scores by projecting data observations onto model planes, which are defined by the extracted principal components. The integrated peak areas, corrected for the fresh weight of the plants, were normalised, i.e. peak areas of all analysed compounds (X variables) were log-transformed (the constant 0.00001 was added to provide non-detectable components with a small non-zero value (Sjödin et al., 1989)) and mean-centered, scaled to unit variance and represented as a matrix X (Eriksson et al., 2001). The objective of PLS-DA is to find an optimal model that discriminates the X data according to the plant treatments (Eriksson et al., 2001). PLS-DA is a supervised technique, so class memberships of the observations need to be predefined. Therefore, an additional Y matrix was made with G columns containing the values 1 and 0 as dummy variables for each of the plant treatments respectively. The number of significant PCs and PLS components were determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001). In addition, we calculated the variable importance in the projection (VIP). Variables with VIP values larger than 1 are most influential for the model (Eriksson et al., 2001; Paolucci et al., 2004).

Results

Parasitoid preference

Preference of three species of parasitoid wasps

Testing the three parasitoid species for their preference of volatiles from herbivore-infested, jasmonic acid-treated or control plants yielded similar results for all species (3 × 2 contingency table test: control vs. JA: $\chi^2 = 2.16$, df = 2, P = 0.340; JA vs. herbivore: $\chi^2 = 0.77$, df = 2, P = 0.681). When control plants were tested against JA-treated plants in the windtunnel *C. rubecula*, *C. glomerata* and *D. semiclausum* all significantly preferred the volatiles from JA-treated plants (binomial test: all $P < 0.001$; Figure 1). When the wasps were presented with a choice between JA-treated plants and plants infested with herbivores, *P. rapae* for *C. glomerata* and *C. rubecula*, and *Pl. xylostella* for *D. semiclausum*,

all three parasitoid species displayed a significant landing preference on the herbivore-infested plants (binomial test: all P < 0.001; Figure 1).

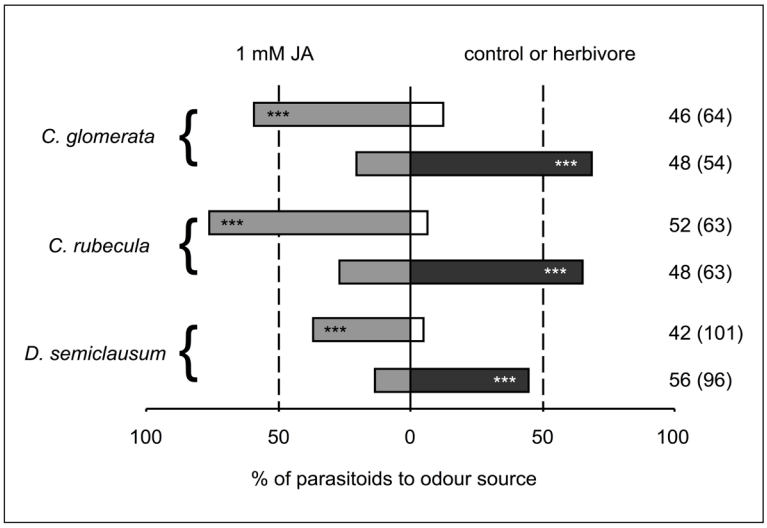

Figure 1: Behavioural responses of three parasitoid wasp species, *C. rubecula, C. glomerata* and *D. semiclausum* when offered two odour sources treated 24 h before testing in a windtunnel. For each parasitoid species the percentage (of the total number of parasitoids tested) of parasitoids that landed on control (white bars) versus 1 mM jasmonic acid (JA)-treated (grey bars) plants, and the distribution of choices for 1 mM JA-treated versus herbivore-infested (black bars) plants are shown. The numbers to the right of each bar represent the number of wasps that made a choice, between brackets the total number of wasps tested (*** P < 0.001).

Preference behaviour of Diadegma semiclausum – without airflow

In still air conditions the preference behaviour of *D. semiclausum* was similar to the preference recorded in the windtunnel (2 × 2 contingency table test: control vs. JA: $\chi^2 = 0.21$, df = 1, P = 0.648; JA vs. herbivore: $\chi^2 = 1.45$, df = 1, P = 0.229). Also in this set-up the JA-treatment of Brussels sprouts plants resulted in a stronger attraction compared to the control treatment (binomial test: N = 39, P < 0.001) and herbivore-infested plants were preferred over JA-treated plants (binomial test: N = 44, P = 0.024). Preliminary results of the same experiment with a fourth parasitoid species, *C. vestalis* (= *C. plutellae*), a specialist parasitoid of *Pl. xylostella*, in the windtunnel as well as in the cage, indicated the same pattern as observed for the other three parasitoid species (results not shown).

Dose-effect relationship

The preference of *C. glomerata* for the volatiles from JA-treated or control plants was tested in dual choice tests in the windtunnel for four concentrations of JA, i.e. 1 µM, 10 µM, 100 µM and 1 mM. Only for the lowest concentration tested the parasitoids did not show a preference for either of the plants (binomial test: N = 21, P = 0.500) (Figure 2). The JA-treated plants were significantly more attractive than the control plant when treated with 10 µM (binomial test: N = 30, P = 0.049), 100 µM (N = 30, P < 0.001) and 1 mM JA (N = 30, P < 0.001). With decreasing concentration of JA, the response level of the parasitoids decreased from around 60% to a response level below 30% for the lowest concentration tested (Figure 2). When considering the separate experimental days, there was a negative correlation between the JA concentration and the percentage no choice (Spearman's rho = -0.671, N = 31, P < 0.001).

Effect of time since treatment

The response of *C. glomerata* to volatiles from JA-treated plants was tested after different time intervals. Already in the first hour after JA application more parasitoids landed on JA-treated plants than on control plants, although the response level in the first three hours after application was very low and the difference therefore not significant (binomial test: $P > 0.05$; Figure 3). After three hours the preference for JA-treated plants was more pronounced and statistically significant (binomial test: $P < 0.001$). The parasitoids still preferred the volatiles from JA-treated plants over the control plants after 120 hours (binomial test: $P < 0.001$). The percentage of wasps that responded, i.e. landed on a plant within 10 minutes, was highest after 24 hours since JA application.

After caterpillar feeding the attractiveness of the plants waned more rapidly (Figure 4). Forty-eight hours after removal of the caterpillars, the plants that had been fed on by 15 caterpillars were still attractive (binomial test: $P = 0.001$), but attractiveness was absent after 120 hours (binomial test: $P = 0.5$). The plants that had been fed on by 5 caterpillars had already lost their attractiveness 48 hours after removal (binomial test: $P = 0.6875$).

Attraction to an inert substrate treated with JA

When JA was offered on an inert substrate the response of the parasitoids was very low. Only 1 out of 34 parasitoids landed on a leaf next to the JA-treated substrate, and none on the substrates. Eight parasitoids flew within a distance of 5 cm of a substrate; six flew near the water-treated paper and two near the JA-treated paper.

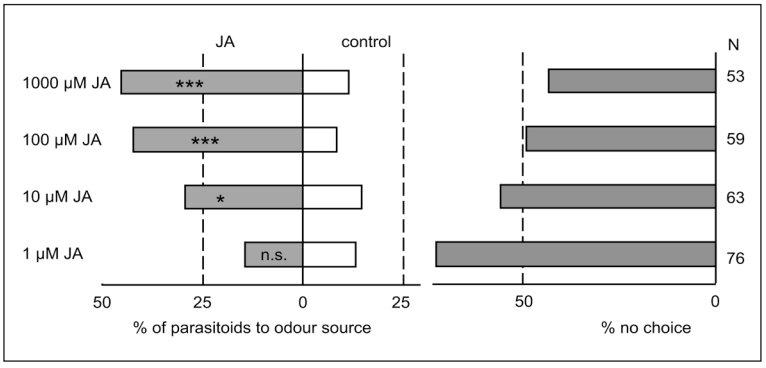

Figure 2. Effect of the concentration of jasmonic acid (JA) applied to Brussels sprouts plants on the attraction of the parasitoid *C. glomerata* in a windtunnel. Plants treated with different concentrations of JA (24 h before the windtunnel test) are tested against control plants. The percentage of wasps that landed on the JA-treated and on control plants is shown (n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$). N indicates the total number of parasitoids tested. The percentage no choice decreased with JA concentration (Spearman rank correlation: $P < 0.001$).

Volatile analysis

We analysed the volatile blends of control, 1 mM JA-treated and herbivore-induced plants. We identified 53 compounds (Table 1). Among the identified compounds were terpenoids, ketones, alcohols, an aldehyde, nitriles, sulphides and esters. The compounds that were produced in highest amounts were (*Z*)-3-hexen-1-yl acetate, sabinene, limonene, 1,8-cineole, β-myrcene and α-thujene (Table 1). JA-treated plants emitted the highest amounts of volatiles, followed by *P. rapae-*

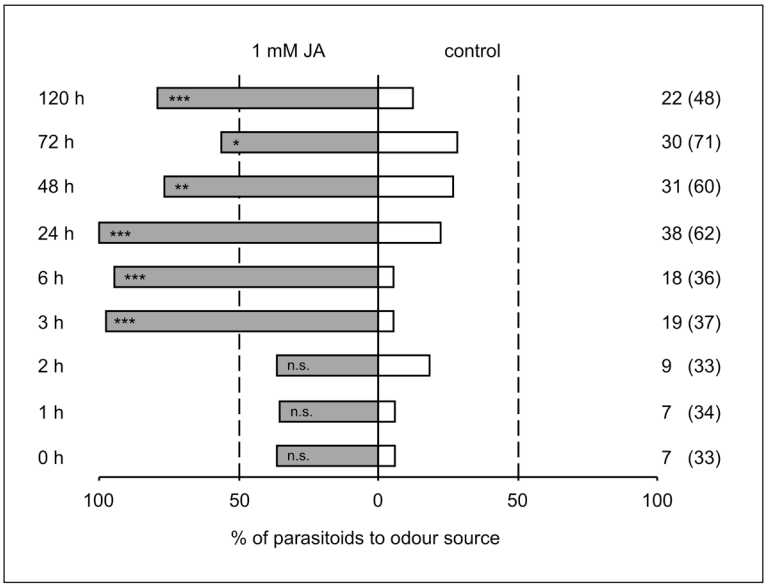

Figure 3. Response of parasitoid *Cotesia glomerata* in a windtunnel at different time intervals since induction of *B. oleracea* plants with 1 mM JA. The percentage (of the total number of wasps tested) of wasps choosing jasmonic acid (JA) or control plants is shown. The numbers to the right of each bar represent the number of wasps that made a choice, between brackets the total number of wasps tested (n.s. $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

infested plants, whereas control plants emitted the lowest amounts and number of volatile compounds. Principal component analysis (PCA) resulted in a model with three significant principal components explaining a total variation (R^2X) of 73%. The score plot shows that the volatile blend composition of *P. rapae*- and *Pl. xylostella*-infested samples slightly overlapped, with *Pl. xylostella*-infested plants showing the lowest degree of within-treatment variation, whereas *P. rapae*-infested plants varied most (Figure 5). The first principal component, explaining 54.9% of the variation of the data, separated control, herbivore- and JA-treated plants, while the second principal component (explaining 10.5%) separated the herbivore treatments from the control and JA-treatment (Figure 5).

To gain more insight into which compounds differ between control, herbivore-damaged and JA-treated plants, we further analysed the data using PLS-DA. PLS-DA showed that the volatile blends of the four treatments are significantly different, as it extracted four PLS components by cross-validation (PLS-DA: 4 PLS components, R^2X (cum) = 0.77, R^2Y (cum) = 0.916, Q^2 (cum) = 0.67); although the fourth component did not add any predictive value to the model. In addition, we calculated the variable importance in the projection (VIP) which is a more numerical value describing the importance of the X variables, both for the X and the Y parts (Wold et al., 1993; Wold et al., 2001). Compounds with a VIP-value > 1 are considered to influence the separation between the groups (Eriksson et al., 2001; Paolucci et al., 2004). In this model 19 compounds had a VIP-value > 1 (Table 1). The most important compounds for separation were (in order of decreasing VIP): (*Z*)-3-hexen-1-yl acetate (1.97), hexyl acetate (1.59), (*E*)-DMNT (1.59), 2-methyl-1-propanol (1.58), and (*Z*)-2-penten-1-ol acetate (1.54). Compounds that have little influence on the separation of the groups were (in order of increasing

VIP): 3-heptanone (0.28), β-myrcene (0.35), limonene (0.36), β-pinene (0.37), α-thujene (0.37), 1-8-cineole (0.38), sabinene (0.39), α-pinene (0.39), and 2-heptanone (0.42).

Discussion

Previous studies documented that caterpillar-infested Brassicaceae are more attractive than control plants for both *Cotesia* species as well as *D. semiclausum* (Blaakmeer et al., 1994a; Geervliet et al., 1994; Van Poecke and Dicke, 2002; Bukovinszky et al., 2005). In this study JA-induced

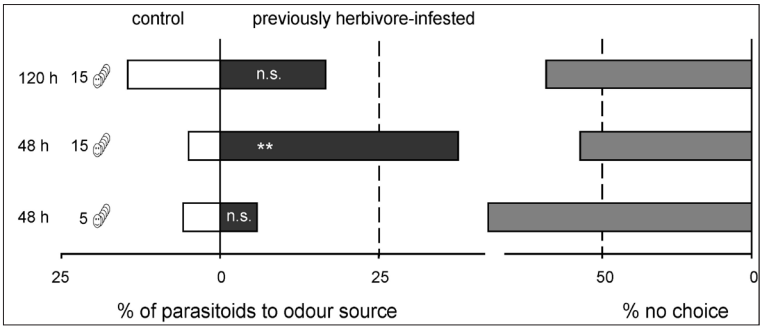

Figure 4. Response of *Cotesia glomerata* wasps in a windtunnel to previously infested plants (black bars) or undamaged plants (white bars) (n.s. $P > 0.05$; ** $P < 0.01$). Caterpillars, *Pieris rapae*, were removed after 24 h of feeding; attraction of the parasitoids was tested 48 or 120 h after removal of the caterpillars. Infestation levels (feeding by 5 or 15 caterpillars per plant) are indicated on the left. Grey bars indicate the percentage no choice in each experiment.

plants were tested against control plants, and all three parasitoid species preferred the volatiles from the JA-induced plants. However, our results also show that herbivore-induced volatiles were more attractive to the parasitoids than the JA-induced volatiles. These results are analogous to the results of Dicke et al. (1999), Van Poecke and Dicke (2002) and Ozawa et al. (2004) for predatory mite attraction to spider mite-induced Lima bean volatiles, and parasitoid attraction to *P. rapae*-induced *Arabidopsis thaliana* volatiles and common armyworm-induced corn volatiles, respectively. This indicates that also in Brussels sprouts plants JA induces part of the plant defence system that is induced by herbivore damage, which renders the plant attractive for parasitoids, but that exogenous application of this hormone can not fully mimic the plant response, indicating the involvement of other factors in the plant defence response. In Lima bean JA induces a similar volatile blend as infestation with the spider mite *Tetranychus urticae,* except for methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) that were not induced and (*E*)-DMNT that was induced in lower amounts by JA (Dicke et al. 1999). Subsequent studies showed that the lack of methyl salicylate induction explained the lower attraction of JA-induced Lima bean plants compared to spider-mite induced plants to predatory mites (De Boer and Dicke, 2004).

In Brussels sprouts plants the JA-induced volatile blend differed from that of the herbivore-infested plants and was less attractive to all three species of parasitoid wasps. The JA- as well as herbivore-induced *B. oleracea* plants emitted higher amounts of (*E*)-DMNT, (*E*)-4-thujanol, hexyl

Table 1 (next page). Volatile compounds detected in the headspace of Brussels sprouts, sprayed with a Tween 20 solution (control, N = 5), infested with either 30 *Pieris rapae* (N = 5), 12 *Plutella xylostella* larvae (N = 4), or sprayed with 1 mM jasmonic acid solution with Tween 20 (N = 4) 24 h before headspace collection. Mean (±SE) of GC peak area (units/gram fresh weight).

	Compound	Control[c]	*Plutella xylostella*	*Pieris rapae*	Jasmonic acid	VIP-values[d]
Alcohols						
1	2-methyl-1-propanol	44.8±16.5	n.d.	74.6±25.2	163.8±58.7	1.58
2	1-penten-3-ol	2.2±2.2	n.d.	194.5±82.0	482.2±101.8	1.48
3	3-pentanol	3.8±3.8	n.d.	60.9±20.7	220.1±42.3	1.42
4	3-methyl-1-butanol	n.d.	n.d.	3.1±2.4	23.5±3.7	0.78
5	1-pentanol	n.d.	n.d.	10.4±4.4	24.9±3.9	0.89
6	(*Z*)-2-pentanol-1-ol	n.d.	n.d.	8.7±4.3	30.7±5.2	0.87
7	3-methyl-2-pentanol	n.d.	n.d.	4.6±2.9	80.5±13.8	0.77
8	(*Z*)-3-hexen-1-ol	157.5±95.4	46.7±8.3	364.8±104.4	902.1±103.3	0.82
9	1-hexanol	5.4±5.4	3.0±3.0	17.5±8.2	61.4±10.4	0.74
10	2-methyl-3-hexen-1-ol	n.d.	n.d.	3.1±2.0	20.4±4.6	0.78
11	3-methyl-3-hexen-1-ol	n.d.	n.d.	3.1±2.0	33.8±16.6	0.78
Aldehydes						
12	hexanal	5.3±3.3	2.3±2.3	6.5±3.4	27.5±18.9	
Esters						
13	3-methyl-1-butanol acetate	n.d.	n.d.	4.1±3.0	19.5±4.7	0.78
14	(*Z*)-2-penten-1-ol acetate	n.d.	20.1±8.4	296.8±154.4	607.8±180.4	1.54
15	pentyl acetate	n.d.	n.d.	29.6±15.0	63.6±20.2	1.14
16	(*Z*)-3-hexen-1-yl acetate	n.d.	364.3±80.9	4249.1±2010.7	9873.6±2934.9	1.97
17	hexyl acetate	n.d.	12.0±2.8	135.9±68.8	605.7±172.7	1.59
18	methyl salicylate	0.9±0.6	2.3±2.3	2.8±1.6	2.0±1.1	0.78
Isothiocyanate						
19	methyl (iso)thiocyanate	n.d.	n.d.	1.3±1.3	25.9±3.6	0.86
Ketones						
20	3-pentanone	n.d.	n.d.	20.6±16.3	456.2±170.3	0.84
21	3-methyl-2-pentanone	n.d.	n.d.	n.d.	169.0±29.0	1.21
22	cyclopropyl-2-propen-1-one	2.7±2.7	1.0±1.0	17.9±8.2	22.2±4.6	0.70
23	3-heptanone	21.4±13.7	13.1±7.1	8.7±2.5	16.0±6.5	0.28
24	2-heptanone	5.9±3.3	5.1±2.7	3.4±1.5	10.1±4.4	0.42
25	2-methyl-2-cyclopenten-1-one	2.7±2.7	n.d.	15.9±6.8	4.0±4.0	1.45
26	2-methyl-6-methylene-1,7-ocatadiene-3-one	n.d.	2.3±2.3	8.3±4.6	55.5±7.0	0.78
Nitriles / N-containing						
27	2-methylbutanenitrile	n.d.	n.d.	n.d.	194.0±49.9	1.21
28	3-methylbutanenitrile	n.d.	n.d.	5.0±3.2	329.9±46.4	0.84
29	benzonitrile	28.1±5.2	14.4±6.3	7.5±6.3	25.3±15.6	0.90
30	benzyl cyanide	4.4±2.7	2.3±2.3	15.9±5.3	40.2±12.3	0.76
Terpenoids						
31	α-thujene	94.6±29.4	112.3±49.5	417.9±65.7	2892.1±261.1	0.37
32	α-pinene	44.0±14.8	51.5±24.2	224.9±20.8	1098.3±40.7	0.39
33	thuja-2,4(10)-diene	n.d.	n.d.	n.d.	111.8±13.3	1.20
34	sabinene	318.4±102.5	733.0±227.8	1851.7±373.8	10996.5±959.3	0.39
35	β-pinene	30.9±8.5	39.4±11.7	148.1±25.0	825.5±43.6	0.37
36	β-myrcene	108.6±32.6	146.0±41.3	396.4±65.3	3448.3±608.7	0.35
37	α-phellandrene	n.d.	n.d.	3.0±3.0	21.0±8.5	0.94
38	α-terpinene	21.5±13.5	2.2±2.2	46.7±27.1	156.1±26.7	0.81
39	p-cymene	10.0±4.0	1.1±1.1	17.3±6.0	92.8±54.4	1.16
40	limonene	294.8±94.4	397.3±96.8	1135.9±200.7	9658.5±1160.4	0.36
41	β-phellandrene	13.3±7.3	8.6±3.2	32.5±10.2	25.3±24.9	0.64
42	1,8-cineole	131.8±39.4	237.5±73.1	704.2±127.0	6110.3±773.4	0.38
43	(*E*)-β-ocimene	n.d.	n.d.	n.d.	49.0±18.4	1.17
44	γ-terpinene	28.6±15.1	2.2±2.2	56.9±30.8	239.5±42.7	1.22
45	(*E*)-4-thujanol	1.6±1.6	13.2±8.1	41.5±13.6	413.0±113.9	1.04
46	terpinolene	8.9±5.9	n.d.	5.5±4.2	81.6±14.3	1.11
47	(*Z*)-4-thujanol	n.d.	n.d.	5.6±3.8	90.0±56.1	0.78
48	(*E*)-DMNT[a]	n.d.	25.1±10.1	84.7±29.3	68.3±5.8	1.59
49	pinocarvone	n.d.	n.d.	n.d.	64.9±13.6	1.18
50	terpinen-4-ol	n.d.	n.d.	n.d.	17.6±5.9	0.91
51	carvone	n.d.	n.d.	n.d.	24.9±9.4	0.93
52	longifolene	12.3±2.2	9.6±4.1	11.5±4.0	20.3±3.7	0.57
Unknown						
53	$C_{10}H_{14}O$, 107,108B[b]	n.d.	n.d.	n.d.	68.5±17.6	1.18
Total amount		1399±433	2225±635	10752±2531	51138±5813	

[a] (*E*)-4,8-dimethyl-1,3,7-nonatriene
[b] numbers indicate ion masses of unknown compounds
[c] n.d.: compound was not detected
[d] VIP: Variable Importance in the Projection for PLS-DA. VIP-values > 1 are most influential for separation of the treatments

acetate, (*Z*)-3-hexen-1-yl acetate and (*Z*)-2-penten-1-ol acetate than control plants, these compounds were also important for the separation of the volatile blends of different treatments, and could therefore be used by parasitoids as host location cues. JA-induced plants differed from herbivore- or non-induced plants mostly in higher emission of several terpenes, such as β-ocimene, thuja-2,4(10)-diene, and terpinene. Previous studies on jasmonate-induced Brassicaceous plant also showed higher amounts of several terpenoids and green leaf volatiles, such as (*E*)-DMNT and (*Z*)-3-hexen-1-yl acetate, compared to non-induced plants (Loivamaki et al., 2004; Ibrahim et al., 2005). JA-induced plants emitted the largest amount of volatiles of all treatments. This suggests that spraying the whole plant with 1 mM JA induces a stronger defence response than infestation with 30 *P. rapae* or 12 *Pl. xylostella* caterpillars for 24 hours. Possibly, JA induced the whole plant, while herbivores were only feeding on small parts of the plant, removing less than 1% of total leaf area, and was mainly locally induced. In *Arabidopsis thaliana*, however, the volatile emission after 1 mM JA application was about 10-fold lower than herbivore-induced volatile emission, but these plants were infested with approximately 15-fold more caterpillars per gram fresh weight compared to infestation rates in this study (Van Poecke et al., 2002). While non-induced Brussels sprouts plants emitted the lowest amounts of volatiles and JA-treated plants emitted the highest amounts of volatiles, the parasitoids preferred the herbivore-induced plants, which emitted intermediate total amounts of volatiles, over JA-induced plants. This implies that not the quantity, but most likely the qualitative composition of the volatile blend is most important for parasitoid attraction. This corresponds to findings on the attractiveness of infested Brussels sprouts and mustard plants to *D. semiclausum* wasps, where the wasps also preferred the plants that emitted lower overall quantities of volatiles (Bukovinszky et al., 2005). Moreover, when the volatiles emitted by JA-treated Lima bean plants were supplemented with MeSA, which is induced by spider mites but not by JA (Dicke et al. 1999), the competitiveness of the JA-induced blend compared to the prey-induced blend was restored (De Boer et al. 2004). Another explanation could be that JA not only induces attractive compounds, but also repellent volatiles that could mask the attractiveness (D'Alessandro et al., 2006) or some compounds become repellent at higher concentrations, as was for example shown for attraction of predatory mites to different concentrations of MeSA (De Boer and Dicke, 2004).

Induced plants emit complex volatile patterns, and parasitoids are often capable of discriminating between odour blends from different plant species, as well as between conspecific plants infested by host and non-host species (Takabayashi et al., 2006). In this study we tested three parasitoid species that differ in host range and specificity. Differences in host specialisation could result in differences in their (innate) response to volatile profiles as well. According to the concept of dietary specialisation and infochemical use, generalist parasitoids use more general cues and specialists more specific cues to locate their host (Vet

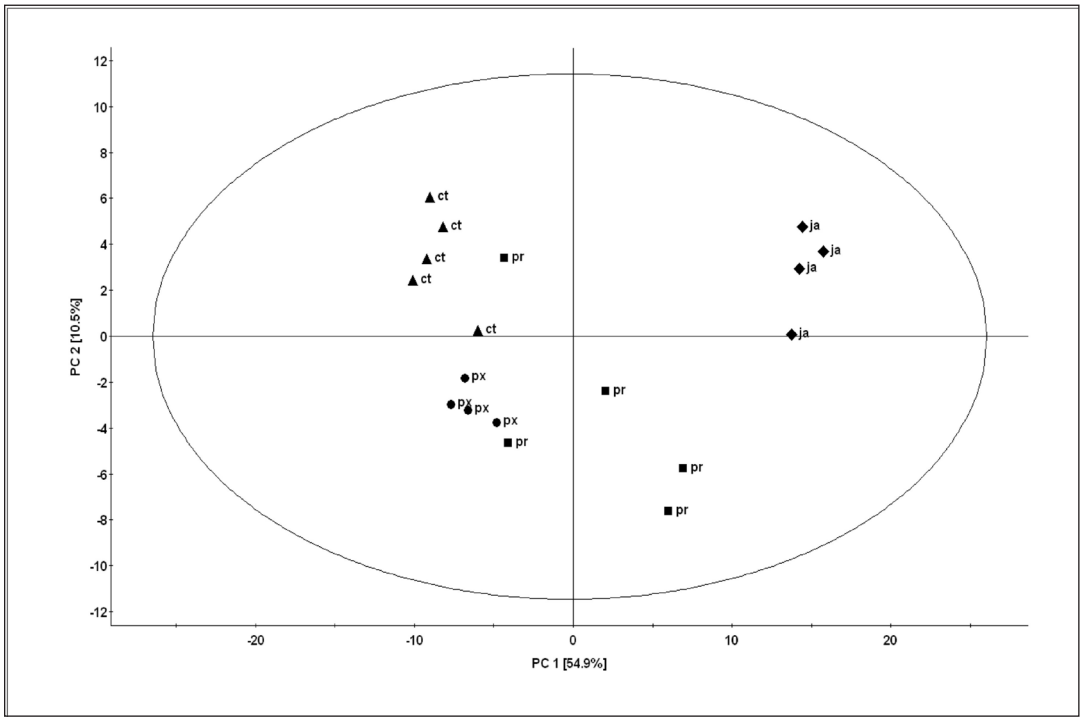

Figure 5. Principal component analysis of the volatile pattern of plants infested with *Plutella xylostella* (px), *Pieris rapae* (pr), jasmonic acid-treated plants (ja) and control plants (ct). First (PC1) and second (PC2) principal components plotted against each other. Percentage variation explained between brackets. The ellipse defines the Hotelling's T^2 confidence region (95 %).

and Dicke, 1992). In an extensive literature study testing this concept, Steidle and Van Loon (2003) show than both generalists and specialists are known to use infochemicals innately, but learning occurs more frequently in generalists. In this study, all three parasitoid species were specialists and responded similarly to the induction by JA, and preferred host-infested plants over the JA-induced ones.

Herbivore-infested cabbage plants already attract *C. glomerata* parasitoids within one hour after infestation (Scascighini et al., 2005). The response increased after the first hour and this preference remained for at least 16 hours. We obtained similar results for the treatment with jasmonic acid, although the response of the parasitoids was very low in the first hours after treatment, i.e. 20–25%. Three hours after JA-treatment the response increased and the parasitoids significantly preferred the JA-induced plants to control plants. The JA-induced defence lasts at least five days after the treatment; after five days the parasitoids still preferred the JA-treated plants to control plants, though the response level of the parasitoids declined slightly with time after 24 hours. The effect of herbivore induction did not last that long; herbivore-damaged plants had lost their attractiveness five days after removal of the caterpillars. Mattiacci et al. (2001) obtained similar results when they tested the attractiveness of leaves excised from herbivore-induced cabbage plants. They found these were attractive to *C. glomerata* one day after removal of the *P. brassicae* caterpillars, but two days after removal the parasitoids did not discriminate between previously infested and control leaves

any longer. Although removal of all herbivores after such as short time interval and JA application are both artificial treatments, these results indicate a difference between herbivore and JA induction. Possibly JA residues remained on the JA-treated leaves that constantly induced the plants, while the induction by caterpillar feeding stopped soon after removal of the caterpillars. In tomato plants in the field increases in polyphenol oxidase and proteinase inhibitors can be measurable three weeks after initial application of JA (Thaler, 1999b) and the number of parasitoid pupae were twice as high on JA-sprayed plants than on control plants after three weeks (Thaler, 1999a).

Comparable concentrations of JA as used here, have been employed in other behavioural, chemical and molecular studies (e.g. Dicke et al., 1999; Koch et al., 1999; Thaler, 1999a; Van Poecke and Dicke, 2002; Zheng et al., 2007). For example, in the same system of Brussels sprouts plants, *Pieris* butterflies preferred to oviposit on control leaves rather than on JA-treated leaves, when treated with 100 µM or 1 mM (Bruinsma et al., 2007, Chapter 3). However, at a concentration of 10 µM the butterflies did not distinguish between treated and control plants, whereas the wasps still did so. In this tritrophic system the parasitoid was more sensitive to induction than its host, possibly because of the use of different cues for host location by herbivores and parasitoids. This indicates that several trophic levels of the insect community can be affected by JA-induced changes in plant. In Chapter 5, we report on studies in which JA is applied to flowering Brassicaceous plants to study the response of another important group of associated arthropods, i.e. pollinators. While nectar secretion was affected by JA treatment, the pollinator visitation did not change after JA treatment.

To eliminate the possibility that the observed attractiveness of JA-treated plants in this study was due to the olfactory perception of JA itself rather than to the induction of plant-produced infochemicals, we tested JA on an inert substrate. Similar to what was demonstrated for the herbivores (Bruinsma et al., 2007, Chapter 3), JA itself did not attract *C. glomerata*. Therefore, we ascribe our results to induction processes in the plant.

JA biosynthesis is suggested to be regulated by positive feedback (Wasternack, 2007). This fits well with the observation that JA application to Brussels sprouts plants induces expression of *BoLOX*, a lipoxygenase gene from *Brassica oleracea* of the octadecanoid pathway upstream of JA, that is also induced by insect-herbivore feeding (Zheng et al., 2007). JA application will therefore not only induce compounds downstream, but also oxylipins and gene expression upstream of JA. OPDA, an intermediate of the octadecanoid pathway has been demonstrated to mediate resistance in *A. thaliana* in the absence of JA (Stintzi et al., 2001) and accumulates in Brussels sprouts plants in reponse to herbivore infestation (Chapter 7). This study shows that also in Brussels sprouts JA-induced processes play an important role in the attraction of parasitoid wasps to the plants. JA-induced plants were attractive compared

to control plants, but even though they emitted more volatiles, the parasitoids were still more attracted to herbivore-induced plants. This shows that parasitoids can discriminate between herbivore induction and artificial induction of plants, and that for actual herbivore induction more factors than JA alone are involved, like the salicylic acid- and ethylene-pathway, and visual cues (reviewed in Dicke and Van Poecke, 2002; and Van Poecke, 2007). Many studies demonstrated a negative effect of salicylic acid (SA) on JA-inducible defences (e.g. Doares et al., 1995; Thaler et al., 2002c; Cipollini et al., 2004). And also ethylene was shown to interact with JA-inducible defences (Kahl et al., 2000; Stotz et al., 2000). This suggests that while JA and other oxylipins play a central role in defence against herbivorous insects, cross-talk between different phytohormones can fine-tune attacker-specific defence responses, which offers interesting possibilities for future research.

Acknowledgements
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Chapter 5

TIBOR BUKOVINSZKY

Differential effects of jasmonic acid treatment of *Brassica nigra* on the attraction of pollinators, parasitoids and butterflies

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Abstract

Herbivore-induced plant defences influence the behaviour of herbivores, as well as that of their natural enemies. Jasmonic acid (JA) is one of the key hormones involved in both these direct and indirect induced defences. JA treatment of plants changes the composition of defence chemicals in the plants, induces volatile emission and increases the production of extrafloral nectar. However, few studies have addressed the potential influence of induced defences on flower nectar chemistry and pollinator behaviour. These have shown that herbivore damage can affect pollination rates and plant fitness. Here, we have investigated the effect of JA treatment on floral nectar production and the attraction of pollinators, as well as the effect on the behaviour of an herbivore and a natural enemy. The study system consisted of black mustard plants, *Brassica nigra* L., pollinators of *B. nigra,* i.e., honeybees and syrphid flies, a specialist herbivore, *Pieris rapae* L. (Lepidoptera: Pieridae), and a parasitoid wasp that uses *Pieris* larvae as hosts, *Cotesia glomerata* L. (Hymenoptera: Braconidae). We show that different trophic levels are differentially affected by JA-induced changes. While the herbivore prefers control over JA-treated leaves for oviposition, the parasitoid *C. glomerata* is more attracted to JA-treated plants compared to control plants. We did not observe differences in pollinator preference, the rates of flower visitation by honeybees and syrphid flies were similar for control and JA-treated plants. Plants treated with JA secreted less nectar than control plants and the concentrations of glucose and fructose tended to be lower than in nectar from control plants. JA treatment resulted in a lower nectar production than actual feeding damage by *P. rapae* caterpillars.

Introduction

Induction of defence responses in plants can alter the behaviour of associated insects. This is well-studied for foliar herbivores (folivores) and their natural enemies associated with vegetative plants (e.g. Dicke et al., 1990a; Turlings et al., 1990; Shiojiri et al., 2002; Bruinsma and Dicke, 2008). However, the effects of induced defences on flowering plants, and consequently pollinator behaviour, have received much less attention. Root and foliar herbivory may indirectly affect plant fitness, by reducing resources for reproduction through reduction of root volume or photosynthetic area. Floral herbivory (florivory) directly affects plant fitness, by reducing the number of gametes (Poveda et al., 2003). Observed effects of foliar herbivory on flowering plants include a decrease in pollinator visitation, an increase in secondary metabolites in leaves, flowers or nectar, fewer and smaller flowers and decreased pollen production (Strauss et al., 1996; Lehtilä and Strauss, 1997; Mothershead and Marquis, 2000; Ohnmeiss and Baldwin, 2000; Hambäck, 2001; Strauss et al., 2004; Smallegange et al., 2007). Root herbivory can increase pollinator visitation (Poveda et al., 2003;2005) and florivory can decrease the number of flowers, nectar production, seed set and pollinator visitation (Krupnick et al., 1999; Adler et al., 2001).

Although these examples show that herbivory can increase or decrease pollinator visitation, depending on type of damage, plant and pollinator species, the mechanisms causing this change in pollinator attraction have not been elucidated (Kessler and Halitschke, 2007). For example, in wild radish both bees and syrphid flies preferred undamaged plants to plants with leaf herbivory (Lehtilä and Strauss, 1997). The preference of the bees could be explained by reductions in number and size of flowers on infested plants. Preference of the syrphid flies for undamaged plants remained when the plants were controlled for these characteristics; indicating another, possibly chemical, basis for differential syrphid fly attraction.

Flowers are not only visited by mutualistic pollinating species. Also herbivore adults such as *Pieris rapae* butterflies may forage on *B. nigra* flowers. Herbivores can be attracted or repelled by volatiles emitted by vegetative parts of the plants; moreover, flower volatiles can influence their foraging behaviour (Honda et al., 1998; Wäckers et al., 2007). Changes in nectar secretion or flower volatile production could therefore not only affect pollinators but also herbivores and natural enemies of the herbivore that also forage on the same flowers. This implies that direct effects on plant fitness due to insect-flower interactions will not only depend on pollination rates, but also on attraction of herbivores and their natural enemies to flowers.

In the present study we investigated the effect of herbivore-induced defences on herbivores, parasitoids and pollinators. We explicitly excluded the effect of physical feeding damage by using a plant hormone to induce

plant defence responses. This method of induction does not remove any tissue and reduces variability due to uncontrollable differences in the amount of feeding damage. This allowed us to compare the response of associated insects to the chemical changes of the plant without any visual cues or indirect effects resulting from tissue removal, which in itself may influence insect behaviour. In response to herbivore infestation, several signal-transduction pathways are induced that result in the production of defensive chemicals (Dicke and Van Poecke, 2002). For both direct and indirect defence against caterpillars the octadecanoid pathway plays an important role in the induction. Treatment of plants with jasmonic acid (JA), a central phytohormone in the octadecanoid pathway, has been shown to affect herbivore and carnivore behaviour, volatile and extrafloral nectar (EFN) production as well as plant toxin accumulation. JA-treatment increases EFN production in several plant species (Heil et al., 2001; Heil, 2004). Whereas, for instance, oviposition by herbivores decreases, carnivores prefer JA-induced plants over non-induced plants (Thaler, 1999a; Thaler et al., 2001). However, the effects of JA-treatment on floral nectar production and on mutualists, like pollinators, have not been studied so far. We address the following questions in this study: (1) Does JA-treatment of *B. nigra* affect herbivore and parasitoid behaviour as it does in several other Brassicaceous plants? (2) Does JA-treatment affect nectar secretion, quantitatively or qualitatively? (3) Does JA-treatment influence the number or duration of visits of pollinators to flowers?

Material and methods

Plants and insects

All experiments were performed with flowering *Brassica nigra* L. (black mustard) plants of ca. 7 weeks old (ca. 1.5 m in height), growth stage 4.2 (Harper and Berkenkamp, 1975), that were grown in a greenhouse at 22-26 ºC, 50-70% r.h. and a L16:D8 photoperiod. The plants were grown from seeds collected in the field in 2005 from *Brassica nigra* accession CGN06619 open-pollinated plants (obtained from the Centre for Genetic Resources, Wageningen, The Netherlands).

A colony of honeybees, *Apis mellifera* L. (Hymenoptera: Apidae), was provided by a commercial beekeeper (Inbuzz, Wageningen, The Netherlands). The hive consisted of three frames with brood of all stages plus the laying queen. The colony was placed in the greenhouse compartment during the days that the experiments were performed (two days a week). The remaining days of the week, the hive was moved to a field outside the greenhouse. In the greenhouse, the bees could forage on *B. nigra* flowers. Outside the greenhouse they foraged on a range of other plant species present in the field.

Small cabbage white butterflies, *Pieris rapae* L. (Lepidoptera: Pieridae) were reared on Brussels sprouts, *Brassica oleracea* var. *gemmifera* L.

cv Cyrus at 22-26 ºC, 50-70% r.h. under a L16:D8 photoperiod. Adult butterflies were fed a 10% sucrose solution and allowed to oviposit on Brussels sprouts plants until the day before the experiment. *Cotesia glomerata* L. (Hymenoptera: Braconidae) was reared on caterpillars of the large cabbage white butterfly, *P. brassicae* L., at 22-26 ºC, 50-70% r.h. and with a L16:D8 photoperiod. Female wasps, eclosed 3 – 7 days before the experiment, that had no previous experience with plant material, were used for the experiments.

Plant treatments

For the JA treatment *B. nigra* leaves were sprayed with a 0.5 mM (±) jasmonic acid (purity >97%; Sigma-Aldrich, St Louis, MO, USA) solution with 0.1% Tween 20 as a surfactant. Both sides of all leaves were sprayed until run-off. The control plants were sprayed with 0.1% Tween 20 solution. On average the leaves were sprayed with 12 µl solution/ cm^2. The JA-treated plants and the control plants were selected for the same height and number of open flowers. Herbivore-infested plants were infested with 2nd instar *P. rapae* caterpillars on the middle three fully expanded leaves, 10 caterpillars per leaf. The plants were used for experiments 48 h after treatment.

Butterfly behaviour

The butterfly bioassay was similar to the one described by Bruinsma et al. (2007) (Chapter 3). One male and one female butterfly were placed in a cage (67 × 50 × 75 cm), in a greenhouse compartment at 22-24 ºC and 50-70% r.h., one day before the experiment. The next morning, one freshly excised control and one JA-treated leaf were introduced into the cages. The middle three fully expanded leaves of a plant were used for these experiments. Butterflies were allowed to oviposit for approximately 4 hours. Subsequently, the number of eggs on each leaf was counted. Apart from natural daylight, the cages were illuminated by sodium vapour lamps (type SON-T, 500 W, Philips, The Netherlands) from 8:00 until 14:00 hours.

Parasitoid behaviour

Parasitoid choice experiments took place in a flight chamber in a greenhouse compartment at 24 ± 2 ºC and 50-70% r.h. with additional illumination provided by six lamps of the same type as used in the butterfly experiments. In the flight chamber, a gauze tent of 293 × 200 cm and 230 cm in height, stood a table (90 cm high) on which a glass cylinder, a JA-treated and a control plant were placed. The female parasitoids were released from a glass cylinder (50 cm above the surface of the table, on a distance of 50 cm from the two plants) on a piece of caterpillar-damaged leaf from which the caterpillars and their faeces had been removed. The JA-treated and control plant were positioned at 50 cm distance from each other. The plant on which the first landing was made within 10 min after release was recorded; no landing on a plant within 10 min was recorded as 'no choice'.

Honeybee behaviour

In order to test whether there are differences in the attraction of pollinators between JA-induced plants and control plants, flower visitation by honeybees was observed in the greenhouse. The experiments were performed between 13:00 and 17:00 hours in the same flight chamber as used for the parasitoid choice experiments, at 22 ± 2 ºC and 50-70% r.h. Four plants, two JA-treated and two control plants, were placed in a square on a table with a surface of 123 cm × 91 cm and a height of 90 cm. Plants of the same treatment were placed diagonally. The distance between plants was approximately 80 cm. A single honeybee at a time was released into the flight chamber with the four plants. Its behaviour was recorded using a handheld computer (Psion Workabout), programmed with The Observer (version 4.1, Noldus Information Technology, Wageningen, The Netherlands). Two parameters of flower visitation behaviour of the bee were recorded for 10 min: the plants it visited and the time it spent on the plants. After this time, the bee was caught and released outside the tent and a new bee was released inside the tent. Bees to be introduced into the setup were caught when they were leaving the hive because they are most likely to be motivated to collect nectar. After two bees had been observed, the plants were rotated to exclude positional effects and after ten bees the set of four plants was replaced with a new set of plants. In total 17 sets of plants were observed.

Field observations

Flower visitation by naturally occurring pollinators was observed in field experiments from late June until mid August 2006. The different species and number of insects that visited the JA-treated plants were observed and compared to the species and number of insects that visited the control plants. Approximately 48 h after JA treatment, the plants were transported from the greenhouse to an agricultural field in the vicinity of Wageningen, The Netherlands. There, they were planted (without pot) in a square (two JA-treated and two control plants per experimental day) 150 cm apart. Other flowering plants in the plot were removed as much as possible.

Every plant was observed twice a day for five min to record the pollinators that visited the plant. Not every individual pollinator that visited the plants could be identified to species. The most common species present in the field belonged to four important pollinator groups: honeybees (*Apis mellifera*), solitary bees, bumblebees (*Bombus* spp.) and syrphid flies (Syrphidae). The pollinators that belong to these categories are relatively easy to discriminate and when an individual entered the plot, it could rapidly be classified in one of these four groups. The pollinators were observed using the same handheld computer with The Observer software as for the honeybee observations in the greenhouse, which was now programmed to record both the arrival and the departure of every single individual of these species groups that entered the plot. The first series of observations took place approximately 48 h after treatment,

on the same day the plants had been planted in the field. The second series of observations took place ca. 96 h after treatment. Sometimes, due to heavy rainfall, a planned series of observations was not possible and then the first observations were made 72 h after treatment when possible.

Nectar collection

To test the effect of JA-treatment on the nectar secretion of *B. nigra,* the quantity and sugar composition of the nectar of JA-treated and control plants were measured. Nectar was collected from the plants 47 ± 1 h after treatment. Around 8:00 am, one hour before collecting the nectar, the air humidity was increased to approximately 80% r.h. using a humidifier (Defensor 3001). The nectar was collected with a capillary from three flowering branches of the inflorescence of every plant: the central, third and fifth flowering branch counted from the top of the plant. Nectar was collected from the 5 distal flowers of every branch. Hence, nectar was collected from 15 flowers of every plant. After the nectar had been collected, the number of open flowers of every plant was counted.

To reach the nectaries more easily the capillaries were adjusted by heating the end of a 5 µl glass capillary tube (Sigma Blaubrana intramark) and elongating it until a thin pointed end was formed using a vertical pipette puller (Narishige, Puller PB). The amount of nectar collected from each plant was determined by measuring the number of millimetres (1.48 mm corresponded to 1 µl) of nectar in a capillary (total amount of nectar collected from 15 flowers per plant). The obtained nectar per plant was stored in an Eppendorf tube with 10 µl 70% ethanol and kept at -20 ºC until further analysis. The sugar composition in the obtained nectar was determined using HPLC analysis. Samples from each experimental day were paired, yielding 24 series (24 control samples and 24 JA-treated samples). The samples were diluted 10 times and injected in a Dionex BioLC system, equipped with a GS50 gradient pump, a CarboPac PA1 Analytical Column 4 x 250 mm with a CarboPac Guard Column 4 x 50 mm, and an ED50 electrochemical detector. The column was eluted with 100 mM NaOH at 1 ml/min and kept at 25 ºC. The amount of glucose, fructose and sucrose were determined in grams per litre using Chromeleon Software version 6.60 (Dionex Corporation, Sunnyvale, CA, USA).

Data analysis

The number of eggs laid by the butterflies on control and JA-treated plants was compared using a paired t-test using SPSS 15.0. The parasitoid data of the different experimental days were pooled and compared using a binomial test in Microsoft Excel. The number of open flowers was tested for differences between treatments using the Mann-Whitney U-test. These statistical analyses were performed with SPSS 15.0. Differences in the amount and composition of the nectar were analysed with a general linear model (GLM, SAS 8.2®), for which the volume of nectar was normalised by natural-log transformation. The

duration of the plant visits of honeybees to JA-treated and control plants were natural-log transformed and compared for effects of treatment, number of visits, number of flowers and amount of nectar using a mixed model with random coefficients (MIXED, SAS 8.2®). Each bee visited the same plant more than once, the times spent on a plant were considered repeated observations carried out on independent subjects (bees). It was assumed that the time spent on a plant was linearly related to the number of previous visits to that plant, where the deviations from the intercept and slope were random, possibly correlated and drawn from a Gaussian distribution. Because the dataset was unbalanced, the Satterthwaite estimation of the degrees of freedom was used. Non-significant interactions were omitted from the model. In addition, the effect of the order of bees per series was tested. Each bee was classified as number one to ten per series and these were compared separately with a t-test, with Bonferroni correction. The data were also tested for correlation between the amount of nectar produced or number of flowers and the flower visitation with a Pearson correlation test. Only the visits of syrphid flies to the plants were tested since other pollinator species did not visit the plots frequently enough to allow statistical analysis. A Kruskal-Wallis test was performed to assess differences between the plant treatments, and the Mann-Whitney U-test to test for differences between the different observation times. A Spearman rank correlation test was performed in order to test for a correlation between amount of secreted nectar and number of syrphid fly visits per observation (SPSS 15.0).

Results

Butterfly oviposition behaviour

The oviposition preference of *P. rapae* females was compared between control and JA-treated leaves. We observed that *P. rapae* females oviposited more on control leaves than on JA-treated leaves (paired t-test: t = 2.112, P = 0.046, N = 24) (Figure 1).

Parasitoid behaviour

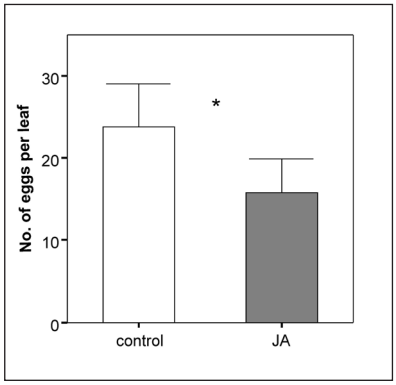

Figure 1. Mean (+ SE) number of *Pieris rapae* eggs on control leaves and jasmonic acid-treated leaves (n = 24, *: P<0.05, paired t-test).

The parasitoid wasps showed a reverse preference. *Cotesia glomerata* females were more attracted to JA-treated plants than to control plants in a dual-choice experiment (binomial test: P = 0.006, N = 48) (Figure 2). However, herbivore-infested plants were preferred both over control (binomial test: P = 0.002, N = 43) and over JA-treated plants (binomial test: P = 0.003, N = 39) by *C. glomerata*.

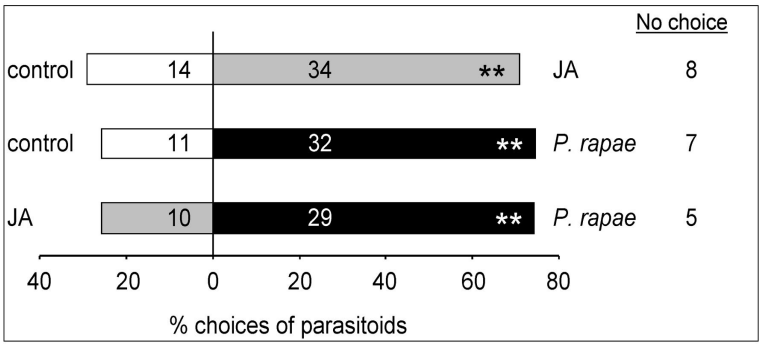

Figure 2. Preference of *Cotesia glomerata* parasitoids for control (white), jasmonic acid-treated (grey) or *Pieris rapae*-infested (black) plants in two-choice bioassays in a flight chamber; numbers in bars indicate numbers of parasitoids, asterisks indicate statistical differences (**: P<0.01, binomial test).

Pollinator behaviour

We did not detect differences in attraction of honeybees to control and JA-treated plants. Moreover, the duration of a visit to a plant did not differ between treatments ($F_{1, 369} = 0.61$, $P = 0.44$). The only factor (negatively) influencing the duration of a visit was the number of visits of a bee to a plant; other factors like the number of flowers of a plant and the amount of nectar secretion did not influence the duration of a visit (number of plant visits: $F_{1,53.3} = 33.29$, $P < 0.0001$; number of flowers: $F_{1,204} = 2.34$, $P = 0.13$; nectar: $F_{1,195} = 0.01$, $P = 0.91$). Furthermore, the number of visits did not correlate with the amount of secreted nectar or number of flowers per plant (number of visits-flowers: Pearson r = -0.081, P = 0.22, N = 236 number of visits-nectar: Pearson r = -0.035, P = 0.60, N = 236). The number of flowers did not differ between treatments (t-test: t = -0.245, P = 0.81, d.f. = 148). However, looking separately at plant visitation by the first bee of each series, we observed a longer visit duration for control plants than for JA-treated plants (t-test: t = 3.977, P = 0.01, d.f. = 16.8). This difference was not observed for any of the subsequent bees (all $P > 0.05$).

In the field the plants were mostly visited by syrphid flies (Table 1), primarily drone flies (*Eristalis tenax* L., Diptera: Syrphidae), a commonly occurring member of the Syrphidae family. None of the three time intervals (48, 72 and 96 h) between plant treatment and pollinator observation, was associated with a difference between the number of visits to control and JA-treated plants (Mann-Whitney U-test: 48 h: Z = -1.138, P = 0.26, N = 82; 72 hours: Z = -0.199, P = 0.84, N = 32; 96 h: Z = -0.623, P = 0.53, N = 96).

Table 1. Total number of pollinators visiting control and JA-treated plants in the field. Each plant was observed for five minutes in an agricultural field near Wageningen from late June to early August.

Species	N of observed individuals	
	Control	JA
Honeybees	1	2
Solitary bees	27	24
Syrphid flies	379	337
Bumblebees	5	1

Nectar analysis

The amount of nectar secreted by *B. nigra* plants that were used for the pollinator experiments was lower for JA-treated plants than for control plants (GLM: treatment: $F_{1, 149} = 4.91$, $P = 0.029$). Since on every experimental day (i.e. same batch of plants and same abiotic conditions) several samples were collected, we included this possible sampling effect in the analysis; and although the amount of nectar differed significantly between experimental days, there was no interaction between experiment and treatment effect (experiment: $F_{23,102} = 4.19$, $P < 0.001$;

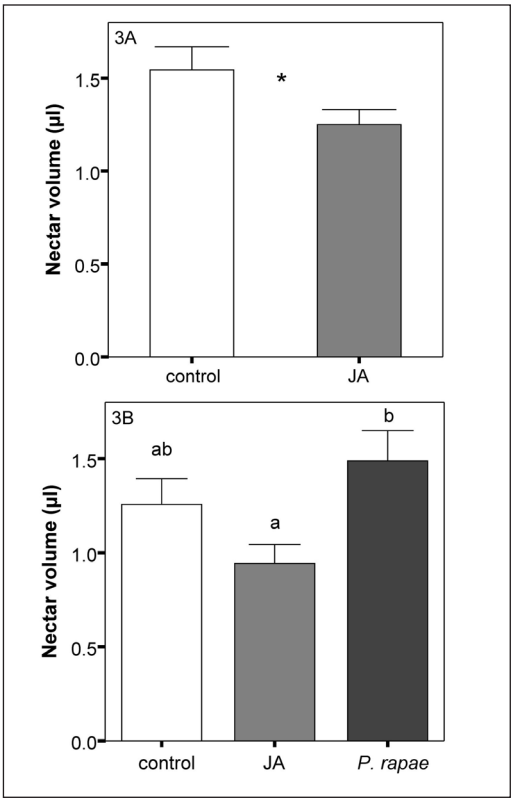

Figure 3. Nectar quantity 48 hours after plant treatment with Tween 20 (control), jasmonic acid (JA), or herbivore-infestation (*P. rapae*). A) Mean (+ SE) of nectar collected during pollinator experiments (GLM, ncontrol = 75, NJA = 75), *: P<0.05; B) Mean (+ SE) of nectar collected in period of parasitoid and butterfly experiments (GLM, Ncontrol = 25, NJA = 22, N*P. rapae* = 18). Significant differences are indicated with different letters.

interaction: $F_{23,102} = 0.76$, P = 0.768) (Figure 3A). In the nectar analysis three compounds were detected: glucose, fructose and sucrose. Sucrose was only detected in 3 out of 48 samples and therefore not included in the statistical analyses. The average concentrations of both glucose and fructose tended to be higher in control samples than in samples from JA-treated plants, the differences being close to significance at the 5% level (GLM: glucose: $F_{1,42} = 3.72$, P = 0.060; fructose: $F_{1,42} = 3.97$, P = 0.053) (Figure 4). We included the amount of nectar as a covariate in the analysis and found that the glucose and fructose concentrations were negatively correlated with the amount of nectar (glucose $F_{1,42} = 11.67$, P = 0.001; fructose: $F_{1,42} = 12.51$, P = 0.001).

In a second series of nectar collection, from the plants that were used for the butterfly and parasitoid experiments, the amount of secreted nectar differed between treatments (GLM: $F_{2,62} = 3.73$, P = 0.030). In this experiment also herbivore-induced plants were included. JA-treated plants secreted less nectar than control and herbivore-infested plants. However, this difference was only statistically significant for JA-treated vs. herbivore-infested plants (Least Squares Means: JA-herbivore-infested: P = 0.023; JA-control: P = 0.248; control-herbivore-infested: P = 0.422) (Figure 3B).

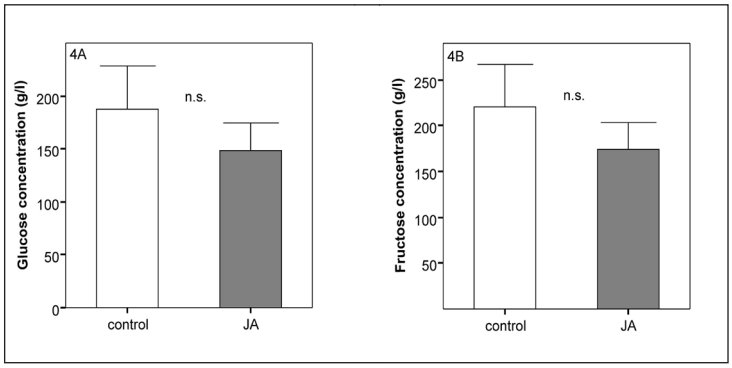

Figure 4. Quality of nectar collected from control plants or plants treated with jasmonic acid (JA) 48 h after treatment. A) Mean (+ SE) concentration of glucose, GLM, P = 0.060, N = 46; B). Mean (+ SE) concentration of fructose, GLM, P = 0.053, N = 46.

Discussion

Treatment of *B. nigra* with JA affected herbivore and parasitoid behaviour. The observation that JA-treatment of plants affects *Pieris* spp. as well as *Cotesia* spp., is consistent with observations made for several other Brassicaceous plants, like Brussels sprouts (Bruinsma et al., 2007; Chapter 4) and *Arabidopsis thaliana* (Van Poecke and Dicke, 2002). These studies observed similar effects of JA dosages comparable to those applied in this study. Although most plants were tested 24 h after treatment, a time series test indicated that a single 1 mM JA application to Brussels sprouts plants can attract parasitoids for at least five days (Chapter 4). These previous studies compared induced and non-induced vegetative plants. In the present study, however, we tested flowering plants. Although we only treated the vegetative parts of the flowering *B. nigra* plants, the flowers could influence the attraction of the parasitoids to the plants, since nectar is an important food source for the parasitoids. However, since the results for vegetative and flowering plants are similar, the presence of flowers does not seem to change parasitoid host location behaviour. In the present study satiated parasitoids were used, as starvation of parasitoids may change their searching behaviour and flowers may be more important to starved parasitoids. Food-deprived *Cotesia rubecula* parasitoids, closely related to *C. glomerata*, prefer flowers over leaves with feeding hosts, while satiated parasitoids prefer the latter over flowers (Wäckers, 1994). Although excised leaves may differ chemically from attached leaves, practical limitations of the setup did not allow testing intact plants with the butterflies. However, because the butterflies discriminated between leaves freshly excised from either JA-induced or control plants, JA-treatment is likely the causal factor (see also control experiments for another *Brassica* species in Chapter 3).

Although nectar volume in JA-treated plants was lower than in control plants, we did not observe any differences in pollinator preference behaviour, except for the first bees of each series. The first bee, of a series of ten, visited control plants longer than JA-treated plants. Since this difference disappeared already with the second bee it is unlikely to be

of great importance in field situations. It remains to be investigated whether pollen or nectar removal by the first bee changed attractiveness. Several studies have shown that herbivory can influence pollinator behaviour. In some of these studies the observed differences could be explained by the difference between the number of flowers of herbivore-induced and non-induced plants (Lehtilä and Strauss, 1997). In our set-up the time interval between spraying and behavioural assays was too short to cause significant differences in flower numbers resulting from treatment. Lehtilä and Strauss (1997) applied the herbivore treatment approximately during two weeks before flowering. When sprayed in an earlier developmental stage, JA may affect time of flowering, as well as the number of flowers (Maciejewska et al., 2004), and therefore may cause differences in pollinator visitation of the plants.

The time interval between induction treatment and behavioural observations was sufficient to allow changes in the amount of nectar secretion. We observed a difference between nectar production in control and JA-induced plants, and between JA-treated and herbivore-infested plants at the herbivore density tested. This means that JA treatment seems to have a different effect than herbivore-infestation on nectar secretion and might therefore not be entirely suitable to simulate herbivore infestation in ecological studies. The effect of JA treatment on parasitoid attraction is, however, similar to that of feeding damage. Moreover, extrafloral nectar production in, for example, *Macaranga tanarius* increased both after JA application and herbivore infestation (Heil et al., 2001). Whether herbivore-infestation would result in changes in the behaviour of pollinators of *B. nigra* plants remains an interesting issue to be investigated.

Besides nectar quantity, sugar concentrations in nectar may possibly influence pollinator visitation rates (reviewed in Mitchell, 2004; Schoonhoven et al., 2005). In the present study the concentrations of both fructose and glucose tended to be higher, but not significantly so, in nectar from control plants than from JA-treated plants. Besides nectar sugars and quantity, other compounds in flowers that may influence pollinator behaviour, such as secondary metabolites, may change in response to stress. Concentrations of secondary metabolites, such as alkaloids, in nectar or flower tissue can increase after herbivory (Euler and Baldwin, 1996; Adler et al., 2006) and result in changes in flower visitation (Gegear et al., 2007; Kessler and Baldwin, 2007). A study on *B. nigra* glucosinolate levels reported a higher level of the dominant compound sinigrin in flower tissues after leaf herbivory (Smallegange et al., 2007). However, we do not know yet whether herbivore damage causes changes in flower visitation, or in secondary metabolites in nectar and floral volatile emission in *B. nigra*. Further studies should clarify the effects of herbivore damage on these plant characteristics; and subsequently elucidate the role of JA in these processes. Timing of induction is also an important factor; treatment of plants in an early

stage of development may have a more severe effect on future flower development than when the plants are already flowering.

For plants that depend on pollination for reproduction it is important to know the effect of induction on pollinator visitation. Enhanced pollinator visitation rates have been shown to increase pollen removal and increase the probability of pollen grains reaching mates (e.g. Galen, 1992), thereby increasing plant fitness, indicating the relevance to investigate effect of infochemicals on pollinator visitation. In this study JA-induced *B. nigra* plants are avoided by *P. rapae* butterflies for oviposition, are preferred by the parasitoid wasp *C. glomerata* over non-induced plants, and although JA treatment reduced the amount of secreted nectar, it did not influence pollinator visitation, suggesting that it is suitable as a crop protectant in this seed crop.

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HANS SMID

Chapter 6

Comparing the effects of the fungal elicitor alamethicin and the phytohormone jasmonic acid on volatile emission by *Brassica oleracea* and on attraction of the parasitoid *Cotesia glomerata*

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Abstract

The induction of plant defences involves different steps with different timing. In this study we compared the effect of induction of an early and a later step of plant defence on plant volatile emission and parasitoid attraction. Ion channel-forming peptides represent a class of elicitors that induce a very early step in plant defence. Alamethicin (ALA) is a peptide mixture from the fungus *Trichoderma viride* that can induce volatile emission and increase endogenous levels of jasmonic and salicylic acid in plants. We used ALA to induce defence responses in Brussels sprouts plants, *Brassica oleracea* var. *gemmifera*, and studied the effect on volatile emission and on the behavioural response of members of the third trophic level to the induced plants. The parasitoid *Cotesia glomerata* was attracted to ALA-treated plants in a dose-dependent manner (dose range 5–50 *μ*g/ml ALA). Jasmonic acid (JA), produced through the octadecanoid pathway, activates a later step in induced plant defence, and JA-induced volatiles are attractive to parasitoids. Treatment with ALA and JA resulted in distinct volatile blends and both blends differed from the volatile blends emitted by control plants. Even though JA treatment of Brussels sprouts plants resulted in higher levels of volatile emission, ALA-treated plants were as attractive to *C. glomerata* as JA-treated plants. Treating plants with a combination of ALA and JA resulted in a volatile blend similar to that after treatment with JA, and rendered the plants more attractive to the parasitoids than treatment with only ALA. We conclude that ALA is a potent inducer of indirect plant defence.

Introduction

Plants are attacked by a variety of herbivores and have evolved a wide range of strategies to defend themselves (Karban and Baldwin, 1997; Agrawal, 1998). Direct defence strategies affect the herbivore itself and indirect defence strategies affect the herbivore by attracting the herbivore's enemies, such as predators or parasitoids (Turlings et al., 1990; Dicke, 1999; Dicke et al., 2003b). Herbivore-induced plant volatiles play an important role in the attraction of predators and parasitoids, which make use of the plant volatiles as cues to locate their prey or host. The emission of plant volatiles can be induced by herbivore feeding and oviposition (Arimura et al., 2005; Hilker and Meiners, 2006; Chapter 2), and has been recorded for more than 23 plant species from 13 families (Dicke, 1999). Signal transduction of herbivore-induced plant defences is mainly mediated by pathways centering around three plant hormones: jasmonic acid (JA), salicylic acid (SA), and ethylene (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Dicke et al., 2003b). Manipulation of the levels of these hormones using elicitors or inhibitors allows investigation of the importance of these hormones for plant responses and insect behaviour in a controlled manner and in the absence of differences in visual cues resulting from feeding damage.

Plant responses to attack can be highly specific. Mechanical wounding elicits a different response than herbivore feeding, and even different herbivore species, herbivore instars and duration of feeding will result in different responses (Bruinsma and Dicke, 2008, Chapter 2). Early events in insect–plant interactions, responsible for recognition of the attacker and triggering signal transduction, take place within the first seconds to minutes after attack, and involve changes in membrane potentials, Ca^{2+}-signalling (spatial and temporal changes in cytosolic Ca^{2+}-concentrations), and production of reactive oxygen species (White, 2000; Maffei et al., 2007a). Oral secretions from eight Lepidopteran larvae (including the herbivores *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella*, that are specialists on Brassicaceous plants and which we studied in Chapters 3, 4, 5, and 7) form ion channels in artificial membranes, and have been suggested to contain compounds that are directly involved in the induction of membrane depolarisation and subsequently in the initiation of defence responses in caterpillar-infested plants (Maischak et al., 2007). Since these events, leading to direct and indirect defence responses, depend on ion fluxes and subsequent intracellular signalling, peptides that produce ion channels within biological membranes can be used to study their potential effect on insect–plant interactions (Engelberth et al., 2000; Maffei et al., 2007a).

Alamethicin (ALA) is a voltage-gated ion channel-forming peptide mixture produced by the fungus *Trichoderma viride*. This mixture consists of at least 12 compounds each containing 20 amino acid residues (Brewer et al., 1987). In Lima bean, ALA treatment increases the levels of both JA and SA. Endogenous levels of JA peak early and transiently

after treatment; SA levels rise more slowly, but remain longer on a high level (Engelberth et al., 2000). Upon treatment with ALA, Lima bean leaves emit an incomplete blend of volatiles compared to treatment with JA; this is probably due to increased levels of SA (Engelberth, 2000) inhibiting the JA response, occurring between 12-oxophytodienoic acid (OPDA, a precursor of JA in the octadecanoid pathway) and JA (Engelberth et al., 2001). Despite of the incomplete blend that is induced, ALA treatment of Lima bean plants results in the attraction of predatory mites to ALA-treated plants, just as does treatment of the plants with OPDA or JA (Dicke and Van Poecke, 2002). ALA is also a potent inducer of MeSA emission in *Arabidopsis thaliana* (Chen et al., 2003), and shares this with the effect of JA on this plant (Van Poecke et al., 2002). It is still unknown whether the volatile release of the model plant in this study, Brussels sprouts, is affected by ALA treatment and whether this affects the behaviour of carnivorous arthropods such as predators and parasitoids.

JA is a key compound in the octadecanoid pathway, involved not only in induced direct defence against herbivorous insects in plants, but also in induced indirect defence (Karban and Baldwin, 1997; Dicke et al., 1999; Thaler, 1999a; Dicke and Van Poecke, 2002; Chapter 3, 4 and 5). Treatment with JA or its volatile ester methyl jasmonate (MeJA) induces a late step of the defence response and renders plants more attractive to carnivorous arthropods in many plant species, including Brussels sprouts (Chapter 4), Lima bean (Dicke et al., 1999; Heil, 2004), gerbera (Gols et al., 1999), tomato (Thaler, 1999a), *A. thaliana* (Van Poecke and Dicke, 2002), tobacco (Kessler and Baldwin, 2001), maize (Ozawa et al., 2004) and rice (Lou et al., 2005). JA-induced plant volatile blends usually contain so-called green leaf volatiles, and terpenoids (Dicke et al., 1999; Van Poecke and Dicke, 2002; Chapter 4). Chemical analysis has demonstrated that herbivory and JA treatment have similar, but not identical, effects on volatile induction in our model plant in this study, Brussels sprouts (Chapter 4), as well as in Lima bean (Dicke et al., 1999; Koch et al., 1999), and *A. thaliana* (Van Poecke and Dicke, 2002). This difference may contribute to the phenomenon that although the predators or parasitoids prefer JA-treated plants to untreated plants, they are more attracted to herbivore-infested plants (Dicke et al., 1999; Van Poecke and Dicke, 2002; Ozawa et al., 2004; Chapter 4).

In this study, we investigated the effect of the induction of a very early step in plant defence signalling, using ALA, and a late step, using JA, by studying volatile emission and parasitoid behaviour. We used the tritrophic interactions between *Brassica oleracea*, *Pieris brassicae*, and *Cotesia glomerata* as a model system to investigate (1) which volatiles are released from Brussels sprouts plants treated by ALA, (2) whether induction by ALA can attract parasitoids, and (3) whether there are interactions between induction by ALA and JA that affect volatile emission and parasitoid attraction.

Materials and methods

Plant and insect material

Brussels sprouts plants, *Brassica oleracea* L. var. *gemmifera* cultivar Cyrus (Brassicaceae) were grown from seeds in a greenhouse in plastic pots (11 x 11 cm) at 24 ± 4 °C, 60 ± 20 % RH and a 16L:8D photoperiod. All experiments were conducted with 5–6 week old plants. The larval parasitoid, *Cotesia glomerata* L. (Hymenoptera: Braconidae), was reared in a greenhouse at 23 ± 1 °C, 60 ± 10 % RH and 16L:8D photoperiod on their preferred host, the large cabbage white butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae). Stock colonies of *P. brassicae* were maintained on Brussels sprouts plants in a climate room at 21 ± 1 °C, 60 ± 10 % RH and a 16L:8D photoperiod.

Plant treatments

ALA was dissolved in methanol at a concentration of 5 mg/ml. From this stock solution the test solutions were prepared by adding water and 0.05% Tween 20 (Sigma-Aldrich, St Louis, MO, USA), resulting in final concentrations of 1, 5, 20 and 50 µg/ml ALA (A&E Scientific, Marcq, Belgium) in the test solution. For the JA treatment, 0.05 or 0.5 mM JA (Sigma-Aldrich, St Louis, MO, USA) aqueous solutions containing 0.05 % Tween 20 and 0.1% methanol were prepared. Concentrations were chosen based on ALA concentrations used previously for *A. thaliana* induction (Dicke and Van Poecke, 2002; Chen et al., 2003) and JA concentrations used for *B. oleracea* in Chapter 4. For the ALA+JA-treatment, the plants were sprayed with an aqueous solution containing six (i.e. 3 ALA × 2 JA) dosage combinations of ALA (5, 20 and 50 µg/ml) and JA (0.05 and 0.5 mM corresponding to respectively 10.515 and 105.15 µg JA/ml), all containing 0.05 % Tween 20.

The upper surface of all leaves with a main vein longer than 4 cm, were rubbed with carborundum powder on a moist cotton pad. Subsequently, the plants were immediately sprayed with 10 ml of a test solution. Control plants were likewise rubbed with carborundum powder, after which the plants were sprayed with 10 ml of an aqueous solution containing 0.05 % Tween 20 and 0.1% methanol. The caterpillar treatment consisted of plants infested with five first-to-second instar larvae of *P. brassicae*. Plants were treated 24 ± 2 hours before use in the experiments.

Preference behaviour of parasitoids

Parasitoid wasp odour preference bioassays were conducted to compare the attractiveness of differentially induced plants in dual-choice experiments. The behaviour of the parasitoid wasps was tested in a windtunnel as described by Geervliet et al. (1994). Three-to-six days old female wasps were used for all experiments and were assumed to have mated. Female wasps were separated from male wasps on the day before the experiment. Before the experiment the wasps were provided with water and honey, but had no experience with plants or caterpillars. The wasps were released individually at approximately 60 cm distance

downwind from the two plants. They were released on a small piece of herbivore-damaged leaf from which caterpillars and faeces had been removed. After release, the parasitoid was observed in the windtunnel until it landed on one of the plants (choice). If the wasp did not land on either plant within 10 minutes, it was recorded as not having made a choice (no choice) and was discarded from the analysis. The position of plants in the windtunnel was alternated after a maximum of five tested wasps to exclude possible directional bias of the set-up. All two-choice combinations were tested on at least five days, with new sets of plants on each day, and each wasp was used only once. The windtunnel conditions were set at 27 ± 1 °C, 65 ± 15 % RH, a light intensity of 24 ± 2 µmol $m^{-2}s^{-1}$ PAR (Quantum meter QMSW-SS, Apogee Instruments Inc., Logan, UT, USA) and a wind speed of 20 cm s^{-1} (Thermisches Anemometer, Wilh. Lambrecht GmbH, Göttingen, Germany). The choices of the parasitoids between two odour sources were statistically analysed using the binomial test.

Alamethicin treatment compared to mechanical damage and herbivore infestation

The behavioural preference of parasitoids to all combinations of control plants, plants treated with alamethicin (20 µg/ml), and herbivore-infested plants was tested in dual-choice tests in the windtunnel.

Dose-response relationship

We tested the effect of different concentrations of ALA on the response of the parasitoids. Plants treated with 10 ml of a solution containing 1 µg/ml, 5 µg/ml, 20 µg/ml or 50 µg/ml ALA were tested against control plants in the windtunnel. The experimental set-up was the same as described above.

Treatment with combinations of alamethicin and jasmonic acid

To test the effect of combinations of ALA with JA, we compared the preference of the wasps for ALA, JA or ALA+JA-treated plants. Six ALA/JA dosage combinations were tested against ALA only, and JA only. Furthermore, ALA treatment and JA treatment were tested against each other at the dosages in which they were mixed. All combinations were tested against each other in dual choice tests in the windtunnel.

Collection of headspace volatiles

For the chemical analysis of volatiles emitted by mechanically damaged Brussels sprouts plants treated either with 0.05 mM JA, 20 µg/ml ALA, 20 µg/ml ALA+0.05 mM JA or control solution, a dynamic headspace collection system was used. A plant of one treatment was placed in a 30 L glass jar. The plastic pot was removed from the plant and replaced by aluminium foil just before the plant was placed in the jar. The jar was tightly closed with a glass lid that was pressed on the jar with a metal clamp with a Viton® O-ring in between. The lid had an air-inlet and an air-outlet. Air was filtered over silica gel, molecular sieve 4Å, and activated charcoal and led into the jar using a vacuum pump. Teflon

tubing was used for all connections. Before the experiments the jars were cleaned with water and ethanol and were then purged with filtered air overnight with a constant flow rate of 100 ml min^{-1}. The flow through the jars was controlled by flow meters (Brooks Instr., Veenendaal, The Netherlands). The system was purged for 1 hour with filtered air before the volatiles were trapped onto the Tenax. Air was sucked out of the jar with 40 ml min^{-1} by passing through a glass tube filled with 90 mg Tenax-TA connected to the air-outlet of the jar. Headspace collections were made in a climate chamber at 23 ± 1 °C, 60 ± 10 % RH, 95 ± 5 µmol m^{-2} s^{-1} PAR. Plant volatiles were collected for four hours. Volatiles of two plants were collected simultaneously, and six replicates per treatment were collected. Six blank controls were taken to determine which compounds were present in the background.

Chemical analysis of headspace volatiles

Headspace samples were analysed with a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer. The collected volatiles were desorbed from the Tenax traps by heating the trap in an automated thermodesorption unit (Ultra; Markes, Llantrisant, UK) at 250 °C for 5 min and flushing with helium at 30 ml min^{-1}. Before thermodesorption, traps were flushed with helium at 30 ml min^{-1} for 3 min to remove moisture and oxygen. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant, UK) at a temperature of 0 °C. Volatiles were injected into the analytical column (RTX-5ms, 30 m x 0.25 mm ID, 1.0 µm – film thickness, Restek, Bellefonte, USA) in splitless mode by ballistic heating of the cold trap for 5 min to 250 °C. The temperature program started at 40 °C (4-min hold) and rose 4 °C min^{-1} to 250 °C (4-min hold). The column effluent was ionised by electron impact (EI) ionisation at 70 eV. Mass scanning was done from 33 to 300 m/z with a scan time of 3 scans s^{-1}. The eluted compounds were identified using Xcalibur software (Thermo, Waltham, USA) by comparing the mass spectra with those of authentic reference standards or with NIST 05 and Wiley library spectra. Linear retention indices were calculated for each compound according to van den Dool and Kratz (1963).

Statistical analysis

The quantitative composition of the volatile mixtures of differently treated Brussels sprouts plants was evaluated by principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) using the software program SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). In PCA, so-called scores are obtained by projecting data observations onto model planes, which are defined by the extracted principal components. Raw data (integrated peak areas corrected for the fresh weight of the plants) were normalised, i.e. peak areas of all analysed compounds (X variables) were summed and the relative amount of each variable was calculated. The normalised data were transformed to log (X + 0.00001). The constant 0.00001 was added to provide non-detectable components with a small

non-zero value (Sjödin et al., 1989). Transformed variables were then mean-centred, scaled to unit variance and represented as a matrix X. The ellipse shown in the score plot defines the Hotelling's T^2 confidence region (95 %). The number of significant principal components was determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001).

The objective of PLS-DA is to find a model that discriminates the X data according to the plant treatments in the best possible way (Eriksson et al., 2001). PLS-DA is a supervised technique, so class memberships of the observations need to be predefined. Therefore, an additional Y matrix was made with G columns containing the values 1 and 0 as dummy variables for each of the plant treatments respectively. The number of significant PCs and PLS components were determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001). In addition, we calculated the variable importance in the projection (VIP) which is a numerical value describing the importance of the X variables, both for the X and the Y parts (Wold et al., 1993; Wold et al., 2001). Variables with VIP values larger than 1 are considered most influential for the model (Eriksson et al., 2001; Paolucci et al., 2004).

Results

Parasitoid preference

Cotesia glomerata females significantly preferred the volatiles from ALA-treated plants to those from mechanically damaged control plants (binomial test, N = 37, P < 0.001; Figure 1). However, the females were significantly more attracted to caterpillar-infested plants when given a choice between caterpillar-infested plants and ALA-treated or control plants (binomial test, N = 35, P < 0.001 and N = 31, P < 0.001 respectively).

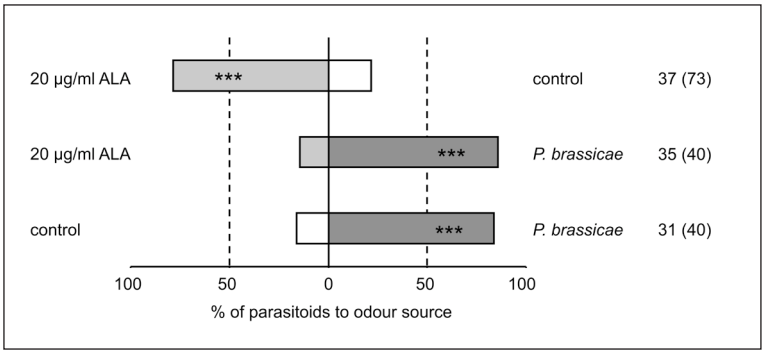

Figure 1. Response of *Cotesia glomerata* females in dual-choice tests in the windtunnel to control plants, plants sprayed with 10 ml of a 20 µg/ml alamethicin (ALA) solution and plants infested with five *Pieris brassicae* caterpillars. The numbers to the right of each bar represent the number of parasitoids making a choice, and the total number of parasitoids used in the windtunnel tests is indicated between brackets (*** P < 0.001).

Dose-response relationship

The wasps significantly preferred the volatiles from plants treated with the three higher concentrations of ALA, i.e. 5 µg/ml (P = 0.008), 20 µg/ml (P = 0.001) and 50 µg/ml (P < 0.001) to the control plants. Only for the lowest concentration tested, 1 µg/ml, the wasps did not display a preference (P = 0.37). When considering the separate experimental days, the percentage of wasps attracted by the ALA-treated plants increased with concentration (Spearman's r = 0.811, N = 25, P < 0.001; Figure 2). The percentage of wasps making no choice in a windtunnel test was not significantly different among the different concentrations (contingency table test: $\chi^2 = 3.961$, df = 3, P = 0.266).

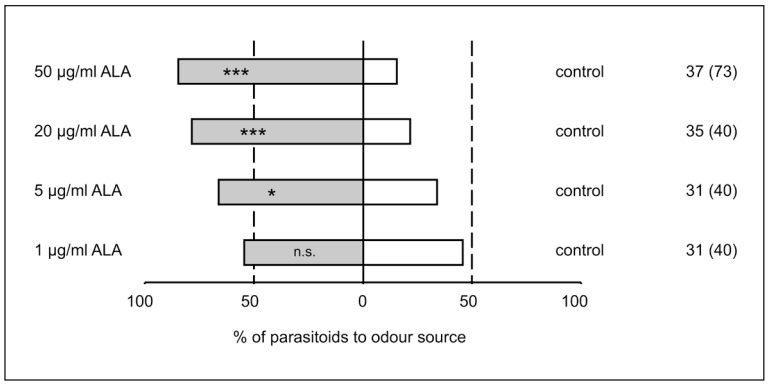

Figure 2. Effect of the alamethicin (ALA) concentration used for treating Brussels sprouts plants on the attraction of *Cotesia glomerata*. The numbers to the right of each bar represent the number of parasitoids making a choice, and the total number of parasitoids used in the windtunnel tests is indicated between brackets (n.s.: P > 0.05; * P < 0.05; *** P < 0.001).

Treatment with combinations of alamethicin and jasmonic acid

The preference of the wasps did not differ significantly between JA- and ALA-treated plants at any combination of concentrations tested (binomial test, P > 0.05; Figure 3). At the low and intermediate concentrations of ALA (5 and 20 µg/ml) in combination with the low JA dose (0.05 mM), the ALA+JA-treated plants attracted significantly more wasps than the ALA-treated plants (P < 0.05), but not more than the JA-treated ones (P > 0.05; Figures 3A–B). In combination with a high JA dose (0.5 mM) the wasps did not prefer the combination to the single treatments (Figures 3D–E). However, at the high concentration of ALA (50 µg/ml), the combination with 0.5 mM JA attracted significantly more wasps than JA alone (P < 0.05; Figure 3F). For the other combinations of JA and the highest concentration of ALA against single compound treatments we observed tendencies for attraction towards the combination of ALA and JA (Figures 3C and 3F).

Volatile emission

We detected 34 compounds in the volatile blends of the four treatments. The blends contained terpenoids, esters, alcohols, an aldehyde, and ketones. Regardless of the treatment, major components of the volatile blends were limonene (21–31% of total blend), 1,8-cineole (14–16%),

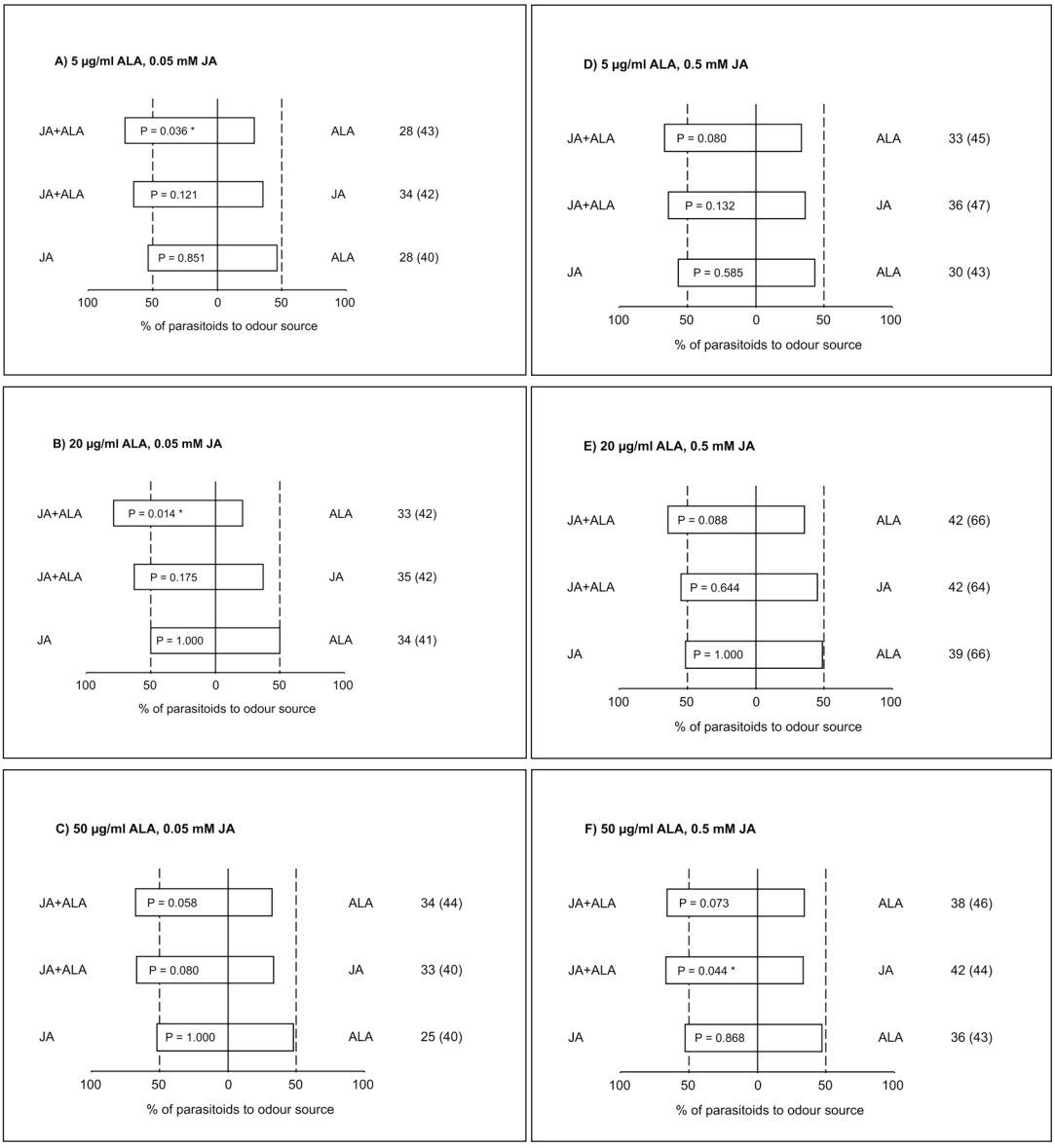

Figure 3. Effect of combinations of alamethicin and jasmonic acid compared with the effects of either elicitor alone on behavioural responses of *Cotesia glomerata* parasitoids in the windtunnel. The numbers to the right of each bar represent the number of parasitoids making a choice, and the total number of parasitoids used in the windtunnel tests is indicated between brackets (* $P < 0.05$).

sabinene (13–15%) and α-thujene (8–21%). The blends from the differently treated plants show quantitative rather than qualitative differences.

A principal component analysis (PCA) based on the relative amounts of 33 compounds (excluding hexanal, because of co-elution with octane) resulted in a model with three significant principal components, explaining 69% of the variation of the data. A plot of the PCA scores of the first two principle components indicates that treating plants with JA or with a combination of JA+ALA induces volatile blends dissimilar from plants sprayed with ALA or control solution (Figure 4). Volatile blends of plants sprayed with JA are similar to those of JA + ALA treated

ones (Figure 4). Volatiles emitted by plants sprayed with ALA showed the largest variation (Figure 4).

We further analysed the data by PLS-DA to determine whether any two treatments differ from each other. Differences in the composition of the volatile blends were significant for all tested combinations, as at least one significant PLS component was extracted by cross-validation; except for the comparison JA vs. JA+ALA which could not be separated (Table 2). For two well-separated groups (G = 2) one would expect G - 1 significant PLS components (Eriksson et al., 2004). More PLS components can indicate subclustering of the volatile blends. The volatile blends of JA and ALA treatments differed significantly in total emission; compounds such as: 2-pentenyl acetate, α-pinene, α-phellandrene, 1,8-cineole, γ-terpinene, α-terpinolene, alloocimene, and (*E*)-DMNT were emitted in higher amounts by JA-treated plants compared to ALA-treated ones (VIP > 1). Compounds with the least influence on the separation of the groups (VIP < 0.5) were: TMTT, 3-pentanone, MeSA and (*Z*)-3-hexen-1-ol (PLS-DA JA vs. ALA).

Discussion

So far, only a few studies have shown that ion channel-forming peptides of fungal origin may represent a novel class of plant defence elicitors of a very early step in the induction process. For example, treatment of *Nicotiana tabacum* with chrysospermin (produced by *Apiocrea* sp.) resulted in increased resistance against tomato mosaic virus infection (Kim et al., 2000) and two peptides from *Trichoderma virens* induced systemic protection against leaf bacteria in cucumber (Viterbo et al., 2007). Alamethicin induces volatile emission in Lima bean (*Phaseolus lunatus*) and *Arabidopsis thaliana* as well as an increase in endogenous levels of plant hormones such as JA and SA (Engelberth et al., 2001; Chen et al., 2003). ALA provides the opportunity to study how induction of a very early step of indirect defence affects the response of carnivorous arthropods. The parasitoid *C. glomerata* responds to herbivore-induced plant volatiles from Brassicaceous plants (e.g. Blaakmeer et al., 1994a; Geervliet et al., 1996). These plant volatiles are more important cues during the parasitoid's host-location behaviour than chemical cues from its host itself or host faeces (Steinberg et al., 1993). *Cotesia glomerata* is also attracted to *B. oleracea* plants that are artificially induced with JA (Chapter 4). In this study, we show that treatment of Brussels sprouts plants with ALA induces the emission of volatiles that attract parasitoid wasps. So far, studies with ALA have not addressed the effect of ALA treatment on arthropod behaviour, except for one experiment performed by M. Dicke and H. Dijkman (described in Dicke and Van Poecke, 2002). They studied the response of predatory mites (*Phytoseiulus persimilis*) to ALA-treated Lima bean plants. Lima bean plants were placed in an ALA solution of 10 µg/ml and subsequently approximately 75% of the predatory mites preferred the volatiles from the ALA-treated plants

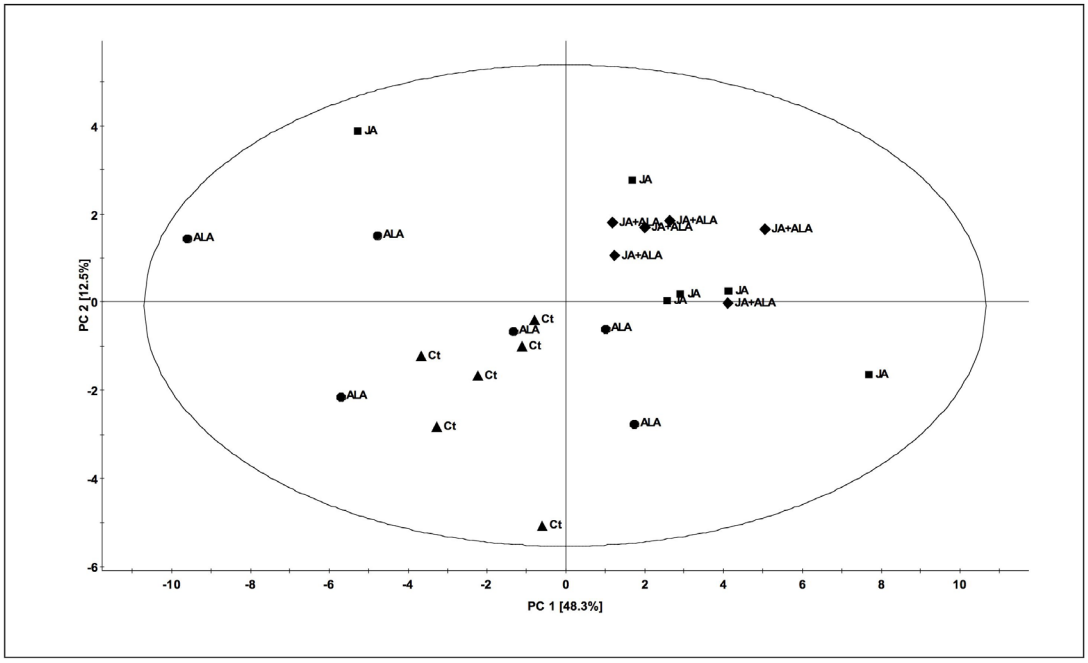

Figure 4. Principal component analysis score plot of the volatile pattern of mechanically damaged Brussels sprouts plants sprayed with Tween 20 (Ct), mechanically damaged Brussels sprouts plants sprayed with 10 ml of a solution of 20 μg/ml alamethicin (ALA), 0.05 mM jasmonic acid (JA) or with a mixture of 20 μg/ml alamethicin and 0.05 mM jasmonic acid (JA + ALA) (N = 6 per treatment). First (PC1) and second (PC2) principal components plotted against each other. Percentage variation explained between brackets. The ellipse defines the Hotelling's T^2 confidence region (95 %).

over those from control plants. The attraction of the predatory mites can be explained with the results from three other studies on the volatile production of Lima bean plants and the use of volatiles by predatory mites. Engelberth et al. (2001) observed an increase, compared tot non-induced plants, in the emission of three major volatiles in Lima bean, TMTT, DMNT, and MeSA, and a trace amount of linalool in response to ALA treatment. Dicke et al. (1990b) and De Boer et al. (2004) observed that these four compounds are important for prey-searching behaviour of *P. persimilis*, which explains why predatory mites are attracted to ALA-treated Lima bean plants.

The parasitoid wasp *C. glomerata* preferred ALA-treated Brussels sprouts plants over control plants in three out of four concentrations tested. ALA treatment of Brussels sprouts plants did not result in higher emission rates of TMTT, DMNT and MeSA as it did in Lima bean (Engelberth et al., 2001). The effect of herbivore induction on volatile emission is different in Brussels sprouts plants and Lima bean plants. Induction of Lima bean plants results in qualitative differences in volatile blend composition while induction of Brussels sprouts plants usually results in quantitative rather than qualitative differences, as was also found in this study (e.g. Dicke et al., 1990b; Mattiacci et al., 1994; Boland et al., 1995). This suggests that induction of volatiles is differently regulated in Lima bean compared to Brussels sprouts plants (Mumm et al., 2008).

Because of the large variation in volatile emission after ALA treatment of Brussels sprouts plants recorded here, it is difficult to determine, based on these results, which compounds are responsible for the difference in

preference of the parasitoids. We do not know whether the parasitoids respond to specific attractive compounds, or to ratios of attractive and repellent compounds, and whether responses increase with concentration above a certain threshold. Several studies suggest that green leaf volatiles, such as (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate, are important for the attraction of *C. glomerata* parasitoids, but also other compounds such as terpenes have been suggested as attractants, and sulphur compounds as repellents (Smid et al., 2002; Scascighini et al., 2005; Shiojiri et al., 2006a; Shiojiri et al., 2006b; Soler et al., 2007). The total volatile emission of JA- and JA+ALA-treated plants was larger than that of control and ALA-treated plants. Possibly, the larger volatile emission of JA+ALA-treated plants is responsible for the preference of the parasitoids for these plants over ALA-treated plants. Yet, a higher volatile emission rate cannot explain the observed parasitoid preference in all tests. An unexpected result in the context of the composition of the volatile blends is the similar response of the parasitoids to ALA- and JA-treated plants. The total volatile emission differed significantly between these two treatments; a range of compounds were emitted at higher rates by JA-treated plants compared to ALA-treated plants (Table 1). However, several compounds were emitted at similar rates in the two treatments; these compounds might be important compounds for the attraction of the parasitoids to the plants. Compounds that occurred in similar amounts and had the least influence on the statistical separation of the groups are TMTT, 3-pentanone, MeSA, and (*Z*)-3-hexen-1-ol.

In Lima bean, both JA and SA were induced by ALA treatment (Engelberth et al., 2000). It is usually thought that JA and other oxylipins play an important role in the induced defence of plants against herbivorous arthropods, whereas SA is mainly involved in the induced defence against pathogens (Karban and Baldwin, 1997; Dempsey et al., 1999; Dicke and Van Poecke, 2002; Van Poecke, 2007). There is growing evidence that the JA- and SA-pathways can negatively interact with each other, e.g. in tomato (Peña-Cortes et al., 1993; Doares et al., 1995; Thaler et al., 2002c), tobacco (Niki et al., 1998; Felton et al., 1999; Rayapuram and Baldwin, 2007) and *A. thaliana* (Gupta et al., 2000; Traw et al., 2003; Cipollini et al., 2004). However, other studies show that the interactions between signalling pathways are not always negative, depending on the dose and timing of elicitor application, and the response measured (Niki et al., 1998; Schenk et al., 2000; Thaler et al., 2002b; Thaler et al., 2002c). For the Brassicaceous plant *A. thaliana* it was shown, using both transgenic plants and exogenous application of JA and SA, that both JA and SA are involved in the induced attraction of the parasitoid *Cotesia rubecula* to *Pieris rapae*-infested plants (Van Poecke and Dicke, 2002).

An increase in SA due to ALA treatment inhibits the octadecanoid pathway between OPDA and JA in Lima bean plants; however, due to the slow increase in SA, inhibition occurs only after several hours, and thus after the typically transient JA burst (Engelberth et al., 2001). If ALA treatment would have a similar effect on Brussels sprouts plants, addition

Table 1. Volatile compounds detected in the headspace of mechanically damaged Brussels sprouts plants sprayed with Tween 20 (control), or mechanically damaged Brussels sprouts plants sprayed with a 10 ml solution of 20 μg/ml alamethicin, 0.05 mM jasmonic acid or with a mixture of 20 μg/ml alamethicin and 0.05 mM jasmonic acid, all three solutions also containing Tween 20 (N = 6 per treatment). Mean ± SE of GC peak area (1000 units/gram fresh weight).

	Compound	Control	Alamethicin	JA	Alamethicin + JA
Alcohols					
1	(*Z*)-3-hexen-1-ol	5.7±3.8	15.2±3.9	31.0±9.9	42.6±4.6
2	1-hexanol	8.7±0.7	11.7±1.7	11.1±2.0	12.9±1.4
Aldehydes					
3	Hexanal[1]	58.9±6.5	61.0±4.4	59.3±7.5	53.3±5.4
Esters					
4	n-butyl acetate	22.0±2.5	22.1±3.9	21.3±4.6	18.7±2.2
5	2-pentenyl acetate	2.5±2.5	9.6±4.4	33.1±7.6	28.3±3.6
6	(*Z*)-3-hexen-1-yl acetate	40.8±16.0	56.9±22.2	390.2±138.0	331.5±85.0
7	hexyl acetate	1.9±1.9	7.0±1.9	22.1±4.4	25.2±4.6
8	methyl salicylate	27.5±8.4	23.3±5.5	21.2±3.4	30.7±6.6
Ketones					
9	3-pentanone	16.0±2.3	22.8±2.4	34.7±11.0	42.6±7.5
10	3-methyl-2-pentanone	2.9±1.2	2.3±0.9	6.9±1.0	6.9±1.3
11	2-hexanone	14.8±2.3	10.8±1.1	11.0±1.9	9.0±2.2
12	3-heptanone	12.5±2.7	9.8±2.5	7.3±1.9	8.4±0.8
13	2-heptanone	6.1±1.1	3.5±0.7	5.0±1.1	4.2±0.3
Terpenoids					
14	α-thujene	256.1±20.0	214.9±35.2	1390.5±1000.8	457.4±48.1
15	α-pinene	52.0±2.4	50.9±4.0	81.8±8.6	78.5±6.2
16	thuja-2,4(10)-diene	4.3±0.4	3.0±1.0	5.1±0.7	3.9±1.0
17	sabinene	460.6±49.2	404.4±78.6	855.1±118.3	834.9±72.8
18	β-pinene	72.3±9.9	59.9±9.1	97.7±10.9	100.7±6.8
19	β-myrcene	107.7±18.0	99.2±25.7	179.0±39.4	215.5±32.9
20	α-phellandrene	27.8±3.0	21.8±4.9	60.7±9.9	52.4±3.4
21	3-carene	9.3±0.6	8.6±0.8	9.9±2.0	9.7±0.8
22	α-terpinene	320.7±203.2	91.7±15.7	206.5±48.7	227.5±15.4
23	limonene	810.6±118.0	885.2±229.7	1369.5±267.4	1379.9±141.8
24	1,8-cineole	485.6±40.6	404.8±71.1	911.0±124.0	906.9±76.2
25	γ-terpinene	114.0±11.2	93.3±20.2	237.1±38.3	226.3±16.8
26	α-terpinolene	108.7±6.7	83.3±11.9	167.3±25.3	165.9±11.0
27	p-mentha-1,8-dien-6-ol, L-carveol	22.8±1.3	22.9±2.4	29.7±3.6	29.1±1.4
28	alloocimene	16.4±0.8	13.0±0.6	22.5±2.5	25.6±2.4
29	(*E*)-4,8-dimethylnona-1,3,7-triene[2]	15.0±4.5	13.6±3.8	31.0±3.8	27.3±3.7
30	d-carvone	9.0±1.6	10.1±2.6	16.7±3.4	14.6±2.2
31	p-cymen-ol	4.5±1.9	4.7±1.5	10.1±1.7	9.0±1.0
32	thymol	9.1±1.1	7.8±2.3	14.4±1.9	12.7±1.8
33	isolongifolene/aromadendrene	14.9±1.2	14.6±1.8	14.3±3.3	13.4±1.2
34	(*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene[3]	14.2±5.5	19.9±5.2	12.8±3.9	31.4±9.1
Total		3248.9±313.0	2884.2±546.3	6473.8±159.8	5543.5±452.3

[1] peak area estimated due to co-elution with octane
[2] DMNT
[3] TMTT

of JA to ALA- treated plants could compensate for the inhibition of the octadecanoid pathway by ALA. In our experiments addition of JA to ALA treatment of plants indeed generally increased the attractiveness of plants to the parasitoids compared to ALA-treated plants, significantly so at two ALA concentrations (5 μg/ml and 20 μg/ml ALA) in combination with 0.05 mM JA (Figure 3) and marginally significantly ($0.058 < P < 0.088$) in the other four combinations. Comparison of the behavioural responses in dual-choice tests with JA+ALA-treated plants versus JA-treated ones, however, did not yield such a clear-cut result. Only in the combination of JA and the highest concentration of ALA (50 μg/ml), ALA increased attractiveness, although less strong at the lower JA concentration (0.05 mM) than at the higer JA concentration (0.5 mM) (Figures 3C and 3F). These data indicate that JA has a more pronounced effect on parasitoid attraction than ALA, although the molar concentrations of JA in this study were higher than those of ALA (50 μg/ml ~ 0.025 mM).

Phenotypic manipulation through the use of fungal elicitors as well as phytohormones can increase our understanding of the induction of plant defence responses and can provide more insight into the use of volatile cues in host searching by carnivorous arthropods. This is clear for the use of ALA in studies with Lima bean, in which ALA induces a qualitatively different volatile blend from control plants, and the induced compounds were shown to be attractive to predatory mites (Dicke et al., 1990b; Engelberth et al., 2001; Dicke and Van Poecke, 2002; De Boer et al., 2004). For Brussels sprouts plants the regulatory network seems to differ from that of Lima bean, and results in quantitative rather than qualitative differences, which complicates uncovering how induction influences carnivores. In this study, ALA treatment induced a volatile blend in Brussels sprouts plants different from that induced by mechanical damage alone. The parasitoids were attracted to the ALA-treated plants, demonstrating that ALA, as an elicitor of an early step in plant defence induction, induces a volatile blend that is attractive to parasitoids. JA treatment resulted in higher volatile emissions than ALA treatment, but resulted in equal attractiveness to parasitoids. A combination of ALA and JA further increased the attractiveness of the plants to parasitoids. Combining different treatments allows comparisons of the relative

Comparison	No. of significant PLS-components	R^2X (cum)	R^2Y (cum)	Q^2 (cum)
ALA vs. Ct	4	0.767	0.992	0.821
ALA vs. JA	2	0.688	0.9	0.722
ALA vs. ALA+JA	4	0.83	0.99	0.882
JA vs. Ct	2	0.667	0.864	0.612
JA vs. ALA+JA	0	0.608	0.64	-0.112
ALA+JA vs. Ct	1	0.597	0.936	0.767

Table 2. PLS-DA results of pairwise comparisons of the head-space of mechanically damaged Brussels sprouts plants sprayed with Tween 20 (Ct), or mechanically damaged Brussels sprouts plants sprayed with 10 ml of a solution of 20 μg/ml alamethicin (ALA), 0.05 mM jasmonic acid (JA) or with a mixture of 20 μg/ml alamethicin and 0.05 mM jasmonic acid (ALA+JA), all three solutions also containing Tween 20 (N = 6 per treatment). Number of significant PLS-components as extracted by cross-validation, total explained variation of the data (R^2X) and predictive power of the model (Q^2).

importance of specific steps of the signal-transduction pathways for both plant chemistry and parasitoid preference.

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TIBOR BUKOVINSZKY

Chapter 4

Effect of the lipoxygenase-inhibitor phenidone on plant response to herbivore feeding and behavioural responses of a parasitoid and three specialist herbivores

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Maarten A. Posthumus, Martin J. Mueller,
Joop J.A. van Loon & Marcel Dicke

Abstract

Herbivore-induced plant defence responses influence behaviour of insects associated with the plant. For biting–chewing herbivores the lipoxygenase pathway has been suggested to play a key role in induced plant defence. We used phenidone, an inhibitor of the enzyme lipoxygenase, to investigate the importance of the early step in this signal-transduction pathway that is mediated by this enzyme. Phenidone treatment of Brussels sprouts plants reduced the accumulation of an internal signalling compound in the octadecanoid pathway downstream of the step mediated by lipoxygenase, i.e. 12-oxo-phytodienoic acid (OPDA). Herbivore feeding induced emission of many volatiles, but after phenidone treatment of the plant, herbivory caused only a slight change in volatile emission. The attraction of *Cotesia glomerata* parasitoids to host-infested plants was significantly reduced by phenidone treatment. The three herbivores investigated, i.e. the specialists *Plutella xylostella, Pieris brassicae* and *Pieris rapae*, showed different oviposition preferences for intact and infested plants, and for two species their preference for either intact or infested plants was shown to be LOX-dependent. The herbivore *Pl. xylostella* prefers infested leaves over intact leaves. Application of phenidone eliminated this preference and infested leaves without phenidone treatment were preferred over infested leaves with phenidone. Contrary to *Pl. xylostella, P. brassicae* prefers intact plants over infested plants for oviposition; but also for this herbivore application of phenidone eliminated the butterflies' discrimination between intact and infested leaves. The third herbivore, *P. rapae*, did not discriminate between intact and infested leaves. Possibly, differences in oviposition strategy cause this difference between the two *Pieris* species. Our results show that phenidone inhibits the defence response of the plant and this inhibition can influence the behaviour of the associated insect community.

Introduction

Insects can use herbivore-induced plant chemicals as cues during host plant selection (Schoonhoven et al., 2005; Bruinsma and Dicke, 2008). Both herbivores and their carnivorous natural enemies can use information on the infestation status of plants to their own benefit. Carnivores search for plants infested with their host or prey, while most herbivores prefer uninfested plants, and thus avoid oviposition on plants that are conspicuous to their enemies and provide competition amongst herbivores. However, in some cases, oviposition on plants infested with heterospecific hosts can be advantageous for herbivores, because it can decrease the searching efficiency of their enemies (Shiojiri et al., 2002).

The octadecanoid pathway has been shown to play an important role in plant responses to caterpillar damage (e.g. Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Arimura et al., 2005; De Vos et al., 2005; Van Poecke, 2007). Lipoxygenase (LOX) is a key enzyme in this pathway and is induced by wounding. The transformation of linolenic acid into 9- and 13-hydroperoxides is catalysed by 9- and 13-LOXs. The hydroperoxides are subsequently converted to aldehydes and oxoacids. Products from 13(*S*)-hydroperoxy linolenic acid can be further transformed by several enzymes to eventually produce jasmonic acid (JA) or mediated by hydroperoxide lyase to (*Z*)-3-hexenal, and subsequenty to (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate (Figure 1) (Koch et al., 1999; Kessler and Baldwin, 2002). LOX-deficient plants are more susceptible to herbivore attack (Royo et al., 1999; Halitschke and Baldwin, 2003; Kessler et al., 2004). Furthermore, caterpillar damage upregulates the expression of a *BoLOX* gene in *Brassica oleracea* (Zheng et al., 2007). The redox-active compound phenidone (1-phenyl-pyrazolidinone, Figure 2) is known to inhibit the activity of LOXs (Figure 1) (Cucurou et al., 1991; Koch et al., 1999; Engelberth et al., 2001), by reducing the active form of LOX to an inactive form. Therefore, phenidone is an effective inhibitor of the octadecanoid pathway, and thus of the plant's induced defence system (Dicke and Van Poecke, 2002).

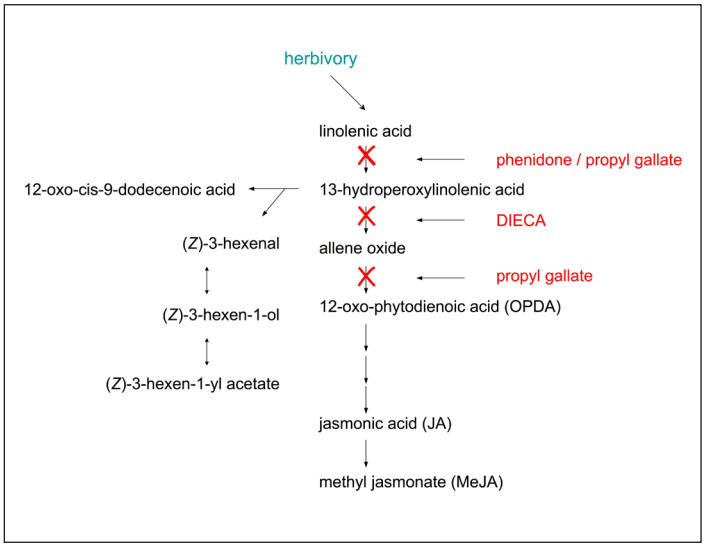


Figure 1: Representation of the octadecanoid pathway from α-linolenic acid (after Creelman and Mulpuri, 2002; D'Auria et al., 2007). Inhibited steps by different inhibitors are indicated.

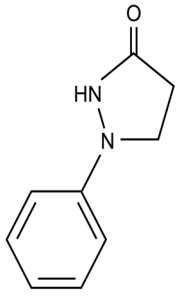


Figure 2. Structure formula of phenidone (1-phenyl-3-pyrazolidinone).

Indeed, several studies found that in Lima bean plants (*Phaseolus lunatus*) phenidone treatment inhibited the emission of volatiles upon treatment with cellulysin, a fungal elicitor of the octadecanoid pathway

(Piel et al., 1997; Koch et al., 1999; Engelberth et al., 2001). Besides plant volatile emission, extra floral nectar (EFN) secretion is also affected by LOX inhibition. Exogenous application of phenidone resulted in a suppression of the response in EFN secretion of nine *Acacia* species, but treatment with JA could restore the EFN secretion (Heil et al., 2004). Furthermore, Kim et al. (2003) observed that LOX activity was not completely inhibited by spraying of plants with phenidone before wounding with a needle, but was delayed from 3 to 6 hours and the expression period was shortened. The inhibitory effect of phenidone is not restricted to LOXs from plants, but phenidone also shows an inhibitory response to LOXs from, for example, human leukocytes (Cucurou et al., 1991; Hlasta et al., 1991).

In the present study we explored whether the inhibitory action of phenidone on lipoxygenase can be used to investigate the effect of this step of the octadecanoid pathway on the behaviour of herbivorous and carnivorous insects. The main question addressed is: is lipoxygenase activity crucial in the expression of direct and indirect plant defence against herbivorous insects? We studied the plant response—oxylipin accumulation and volatile emission—to treatment with different combinations of caterpillar infestation and inhibitor application, as well as the preference of several herbivores and their associated natural enemies for these differently treated plants.

Materials and methods

Insect and plant material

Brussels sprouts *Brassica oleracea* L. var. *gemmifera* cv. Cyrus were grown from seeds in plastic pots (11 × 11 cm) in a greenhouse at 20–28 °C, 40–80% RH and a 16L:8D photoperiod. The large cabbage white, *Pieris brassicae* L., the small cabbage white, *Pieris rapae* L. (Lepidoptera: Pieridae), and the diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) were reared on Brussels sprouts plants in a climatised room at 20–22 °C, 50–70% RH and a 16L:8D photoperiod. The parasitoid wasp *Cotesia glomerata* L. (Hymenoptera: Braconidae) was maintained on *P. brassicae* feeding on Brussels sprouts plants in a greenhouse at 22–24 °C, 50–70% RH and a 16L:8D photoperiod. Adult wasps emerged in a cage without any plants or hosts, were provided with honey and kept at the same climatic conditions as the rearing until use in the experiments.

Plant treatments

Six-to-seven weeks old plants were sprayed with 15 ml of a 2 mM aqueous solution of the inhibitor phenidone with 0.1% Tween 20 (1-phenyl-3-pyrazolidinone and polyoxy-ethylenesorbitan monolaurate, respectively; both obtained from Sigma-Aldrich, St Louis, MO, USA) until run-off. After 30 minutes 15 *P. brassicae* or *P. rapae* second instar larvae were placed on three leaves of the plant i.e. five caterpillars per leaf. As

controls, plants were treated with a 0.1% Tween 20 solution and after 30 minutes infested with 15 *P. brassicae* or *P. rapae* larvae to induce a full volatile blend; or plants were treated solely with the inhibitor solution. After 24 hours at 22–24 °C, 50–70% RH and a 16L:8D photoperiod, the plants were used in the bioassays.

The response of *C. glomerata* was also tested after applying two other inhibitors of different steps of the same signalling pathway: diethyldithiocarbamic acid (DIECA) and propyl gallate (3,4,5-trihydroxybenzoic acid propyl ester; both obtained from Sigma-Aldrich, St. Louis, MO, USA). DIECA reduces 13-hydroperoxylinolenic acid to its corresponding alcohol 13-hydroxylinolenic acid, which is not a signalling intermediate and thus cannot be converted into JA (Farmer et al., 1994; Piel et al., 1997; Bowles, 1998). Propyl gallate is a less specific inhibitor inhibiting both LOX and allene oxide cyclase (AOC), an enzyme mediating the step to 12-oxo-phytodienoic acid (OPDA) in the octadecanoid pathway (Todd et al., 1990; Peña-Cortes et al., 1993; Koch et al., 1999). For both inhibitors 2 mM aqueous solutions with 0.1% Tween were applied to the plants, and tested in the same way as phenidone-treated plants. Because phenidone treatment of plants showed the most clear-cut effect on the attraction *C. glomerata*, the experiments with herbivores and plant measurements were performed only for phenidone treatment.

Bioassays with *Cotesia glomerata*

To determine whether the application of the inhibitors to infested plants changed the attractiveness of the plants for *C. glomerata*, we performed dual-choice windtunnel tests (as described by Geervliet et al, 1994). In the windtunnel for each inhibitor, a plant infested with *P. brassicae* and treated with the inhibitor was tested (1) against a plant infested with *P. brassicae* without inhibitor and (2) against an uninfested plant treated with the inhibitor. All combinations were tested on at least five different experimental days and position of the plants was switched after five wasps to avoid any possible directional bias. Naïve wasps were used when they were 4–7 days old. Female wasps were separated from the males on the day before the experiment. They were released individually in the windtunnel on a piece of leaf from a previously infested Brussels sprouts plant from which all caterpillars, their excreta and silk had been removed just prior to the experiment. The release point was at approximately 60 cm downwind from the two plants. The wasps were observed until they landed on one of the plants. When a wasp did not land on a plant within 10 minutes, this was recorded as no-choice, and the wasp was discarded from the analysis. The windtunnel conditions were set at 25–27 °C, 60–80% RH, light intensity of 24 ± 2 μmol m^{-2} s^{-1} PAR (Quantum meter QMSW-SS, Apogee instruments inc., Logan, UT, US) and a wind speed of 20 cm s^{-1} (Thermisches anemometer, Wilh. Lambrecht GmbH, Göttingen, Germany).

Oviposition preference of *Pieris rapae* and *Pieris brassicae*

Adult butterflies emerged from pupae in a cage of 67 × 100 × 75 cm in a greenhouse compartment at 22–24°C and 50–70% RH. Artificial light (sodium vapour lamps, type SON-T, 500W, Philips, The Netherlands) was used in the cage from 8.00 am until 2.00 pm in addition to natural daylight. The butterflies were provided with a 10% sucrose solution to feed on and a Brussels sprouts plant for oviposition. One day before an experiment started, one male and one female butterfly were introduced in an experimental oviposition cage measuring 67 × 50 × 75 cm. They were provided with sucrose solution to feed on. Two leaves, freshly excised from plants belonging to two different treatment groups were introduced into the cages at 8.30 am and the butterflies were allowed to oviposit until 2.00 pm. Subsequently, the leaves were removed from the cages and the number of eggs on each leaf was counted. The experiment was performed using 10 cages per day and each treatment was replicated 20–30 times. Each day, new pairs of butterflies and new plants were used.

First, leaves infested with caterpillars were tested against uninfested leaves, to test whether the butterflies discriminated between them. The leaves were infested with conspecific larvae. Subsequently, for *P. brassicae* we tested (1) infested leaves with and without phenidone and (2) phenidone-treated leaves with and without caterpillars, the same comparisons as were tested for the parasitoids. Brussels sprouts plants were treated the same way as for the parasitoid experiments and tested 24 hours after treatment, except that freshly excised leaves were used instead of whole plants, with the leaf petioles placed in a vial with tap water.

To test the effect of pure phenidone on the oviposition preference of *P. brassicae* butterflies, intact plants were sprayed with either phenidone or control solution and subsequently the preference of *P. brassicae* was tested 24 hours later.

Oviposition preference of *Plutella xylostella*

Plutella xylostella prefers to lay eggs on cabbage leaves infested with *P. rapae* caterpillars over uninfested leaves (Shiojiri et al., 2002; Poelman et al., in prep). We tested whether this preference could be modified by inhibiting LOX. The set-up of the experiments was copied from Poelman et al. (in prep). One male and one female moth were placed in a plastic cylinder (diameter 13.5 cm, height 21 cm) with two excised leaves that had been treated 24 hours before. The females were allowed to oviposit overnight, and the number of eggs on each leaf was counted the next morning. We first tested leaves from an infested plant against leaves from an intact plant. Subsequently, we tested leaves from plants treated with phenidone and infested with 15 *P. rapae* caterpillars against leaves from intact plants treated with phenidone. Both infested (locally damaged leaves) and systemic leaves (leaves without damage, but from a damaged plant) from these plants were tested. As a final comparison we

tested leaves from two infested plants against each other, one of which was spayed with Tween 20 and the other with phenidone solution.

OPDA analysis

For OPDA analysis leaf material was sampled from plants of four treatments: (1) plants with 15 *P. rapae* caterpillars, (2) plants with 15 *P. brassicae* caterpillars, (3) plants sprayed with 2 mM phenidone and infested with 15 *P. rapae* caterpillars, and (4) plants sprayed with 2 mM phenidone and infested with 15 *P. brassicae* caterpillars. Leaf samples were immediately frozen in liquid nitrogen after sampling and subsequently stored at -80 °C until analysis. For OPDA analysis frozen plant material (ca. 200 mg fresh weight) was transferred into a 2 ml vial. After addition of a ceramic bead (6 mm diameter), tissue was homogenised with a vibrating ball mill (20 s^{-1}, 3 min). Methanol (1 ml) and 50 µl acetic acid were added and the mixture was homogenised again (30 s^{-1}, 3 min). After centrifugation (10 min, 14.000 rpm, Centrifuge 5415C; Eppendorf, Hamburg, Germany), 800 µl of the supernatant was transferred to a 1.5 ml Eppendorf cup and dried in a vacuum centrifuge (Speed-Vac, Christ RVC 2-18). As internal standard, 171 ng $^{18}[O_2]$-12-OPDA (in acetonitrile) was added. The organic solvent was dried under a stream of nitrogen and the residue was dissolved in 100 µl acetonitrile. After centrifugation (10 min, 14,000 rpm), 90 µl was transferred to a new vial and taken to dryness under a stream of nitrogen. The residue was dissolved in 20 µl acetonitrile and transferred to a microvial. Prior to HPLC-MS analysis, 80 µl of ammonium acetate (1 mM; pH 6.6) was added. An injection volume of 10 µl was used for HPLC-MS analysis. Analysis was carried out on a Waters/Micromass (Milford, MA, USA) Quattro Premier Triple Quadrupol mass spectrometer coupled to a Agilent 1200 Series (Agilent, Waldbronn, Germany) HPLC system, equipped with a 1200 Binary Pump and 1200 Standard autosampler. A pre-column (Purospher Star 18e, 4 × 4 mm, 5 µm particle size (Merck, Darmstadt, Germany) and Purospher Star RP 18e column (125 × 2 mm, 5 µm particle size; Merck, Darmstadt, Germany) were used. The injection volume was 10 µl, and the HPLC flow rate was 0.2 ml min^{-1} using the following gradient of ammonium acetate (1 mM, pH 6.6) : acetonitrile mixtures: ten min 95 : 5, five min 5 : 95, then at a flow rate of 0.3 ml min^{-1} 15 min 95 : 5. Mass spectra were acquired using electrospray ionisation in negative ion mode and Multiple Reaction Monitoring (MRM). The capillary and cone voltage were set at 3.00 kV and 40.00 V, the flow rates of cone gas and desolvation gas were 50 and 800 L/hour, and the source temperature and desolvation temperature were 120 and 400 °C, respectively. Data were acquired with MassLynx 4.1 software. Quantification of the compounds was performed by integration of the peak area in the MRM chromatograms. Using an oxygen-18 labelled standard, the concentration was calculated by reference to the integrated area of the isotopic analogue.

Volatile analysis

Volatiles were collected from plants (1) sprayed with phenidone, (2) sprayed with phenidone and subsequently infested with 15 *P. brassicae*, and (3) sprayed with Tween 20 and then infested with 15 *P. brassicae*. The headspace collection was performed in a climate room at 22-24°C, 50-70% RH and a light intensity of 95 ± 5 µmol $m^{-2}s^{-1}$ PAR (Quantum meter QMSW-SS, Apogee instruments inc., Logan, UT, USA). Pressurised air was filtered over silica gel, a molecular sieve (4Å) and activated charcoal, and led through a 30 l clean glass jar. Overnight, clean air was led through the jar at a flow rate of 100 ml/min to remove any remaining volatile contaminants. Just before placing the plant in the jar, the pot of the plant was removed and the roots and soil were packed tightly in aluminium foil. The plant was placed in the jar, which was closed with a glass lid with a Viton® O-ring in between and the lid was tightly closed with a metal clamp. The jar with the plant was purged for 1 hour with an air flow through the jar of 50 ml/min. Subsequently, headspace volatiles were collected at the outlet of the jar on a glass tube filled with 90 mg Tenax-TA 25/30 mesh for 4 hours at a flow rate of 40 ml/min. After collection the tube was closed and stored at room temperature until GC-MS analysis. Two plants of different treatments were sampled at the same time, and five or six replicates per treatment were sampled and analysed. Headspace samples were analysed with a Varian 3400 GC connected to a Finnigan MAT 95 MS. The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack) at 250° C for 10 min and flushing with helium at 14 ml/min. The released compounds were cryofocused in a cold trap (0.52 mm ID deactivated fused silica) at a temperature of -85°C. By ballistic heating of the cold trap to 220°C the volatiles were transferred to the analytical column (DB-5ms J&W, Folsom, CA, 60 m x 0.25 mm ID, 0.25 µm - film thickness). The temperature program started at 40°C (4-min hold) and rose 5°C min^{-1} to 280°C (4-min hold). The column effluent was ionised by electron impact (EI) ionisation at 70 eV. Mass scanning was done from 24 to 300 *m*/*z* with a scan time of 0.7 s/d and an interscan delay of 0.2 s. Compounds were identified by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index.

Statistical analysis

Data on parasitoid behaviour in response to the same plant treatments and obtained on different days were pooled and analysed with a binomial test. Herbivore oviposition preference was tested, depending on the distribution of the data, with a paired t-test or a Wilcoxon matched-pair signed-ranks test. OPDA levels were compared with a Mann-Whitney U test in SPSS 15.0. The volatile patterns of differently treated plants were analysed using Principal Component Analysis (PCA) and Projection to Latent Structures-Discriminant Analysis (PLS-DA) using the software program SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). PCA obtains so-called scores by projecting

data observations onto model planes, which are defined by the extracted principal components. The integrated peak areas, corrected for the fresh weight of the plants, were normalised, i.e. peak areas of all analysed compounds (X variables) were log-transformed (the constant 0.00001 was added to provide non-detectable components with a small non-zero value (Sjödin et al., 1989)) and mean-centred, scaled to unit variance and represented as a matrix X (Eriksson et al., 2001). The objective of PLS-DA is to find a model that discriminates the X data according to the plant treatments (Eriksson et al., 2001). PLS-DA is a supervised technique, so class memberships of the observations need to be predefined. Therefore, an additional Y matrix was made with G columns containing the values 1 and 0 as dummy variables for each of the plant treatments respectively. The number of significant PCs and PLS components were determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001). In addition, we calculated the variable importance in the projection (VIP). Variables with VIP values larger than 1 are most influential for the model (Eriksson et al., 2001; Paolucci et al., 2004).

Results

Bioassays with *Cotesia glomerata*

Pieris brassicae-infested plants treated with phenidone were less attractive to *C. glomerata* than infested plants treated with control solution (binomial test, N = 42, P = 0.008). However, infested plants treated with phenidone were still more attractive than intact plants sprayed with phenidone (binomial test, N = 39, P < 0.001). The inhibitor DIECA showed a similar result; infested plants treated with DIECA are less attractive to *C. glomerata* than infested plants treated with control solution, but are more attractive than uninfested plants treated with DIECA (binomial test, N = 46, P = 0.026 and N = 26, P < 0.001, respectively). Treatment with propyl gallate resulted in lower

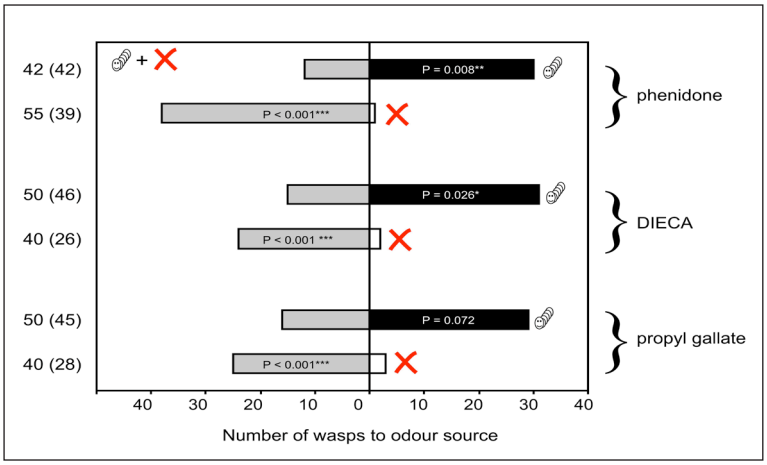


Figure 3. Attraction of *Cotesia glomerata* to plants sprayed with the inhibitors (✕) phenidone, DIECA, or propyl gallate, or sprayed with a control solution, with or without infestation with *Pieris brassicae* (🐛). Numbers to the left of the bars indicate the total number of parasitoids tested, numbers between brackets the number of parasitoids that landed on a plant (binomial test, *** P < 0.001, ** P < 0.01, * P < 0.05).

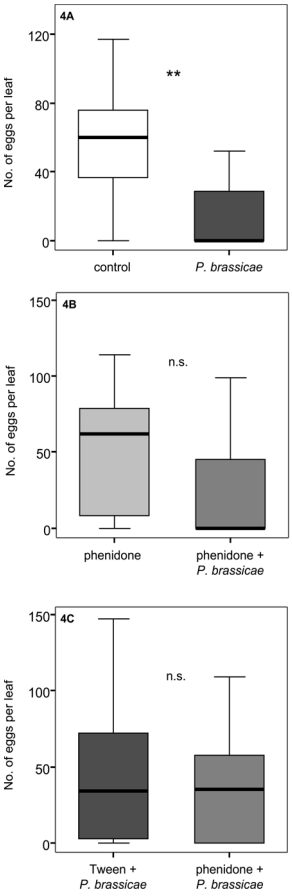


Figure 4. Oviposition preference *Pieris brassicae* on A) *P. brassicae*-infested vs. uninfested (control) leaves, B) *P. brassicae*-infested leaves with or without phenidone C) phenidone-treated leaves with or without *P. brassicae*. The thick line indicates the median, the box represents the interquartile range from first to third quartile (Wilcoxon matched pair signed rank test, ** $P < 0.01$, n.s. not significant).

attractiveness of infested inhibitor-treated plants compared to infested control plants, but not significantly so (binomial test, N = 45, P = 0.072), and propyl gallate-treated infested plants were more attractive than propyl gallate-treated intact plants (binomial test, N = 28, P < 0.001; Figure 3).

Oviposition preference of *Pieris brassicae*

For the herbivores we first assessed oviposition preference for infested vs. uninfested leaves. *Pieris brassicae* discriminated between infested and uninfested leaves, and preferred uninfested over infested leaves (Wilcoxon matched-pair signed-ranks test: Z = -3.244, N = 31, P = 0.001; Figure 4A). When infested plants were treated with phenidone, however, the difference disappeared, although there still was a tendency towards preference for uninfested plants (Wilcoxon matched pair signed ranks test: Z = -1.894, N = 33, P = 0.058; Figure 4B). When infested leaves pre-treated with phenidone or control solution were compared, the butterflies did not prefer one treatment over the other (Wilcoxon matched pair signed ranks test: Z = -0.573, N = 36, P = 0.573; Figure 4C). Phenidone treatment of intact plants did not affect *P. brassicae* oviposition behaviour: the butterflies did not discriminate between plants treated with either phenidone or control solution (Wilcoxon matched pair signed ranks test: Z = -0.211, N = 22, P = 0.842).

Oviposition preference of *Pieris rapae*

Pieris rapae did not discriminate between infested and uninfested plants, for the experiments with 15 caterpillars per plant and 24 hours feeding, although a tendency was seen (paired t-test: t = 1.797, df = 42, P = 0.079). Therefore, phenidone was not expected to have any effect on the oviposition preference of *P. rapae*. When the amount of damage was increased, either by a three-fold increase in caterpillar density, or prolonging the feeding time to a week, the butterflies did discriminate (Wilcoxon matched pair signed ranks test: Z = -3.531, N = 24, P < 0.001; and Z = -2.799, N = 24, P = 0.004 respectively).

Oviposition preference of *Plutella xylostella*

In contrast to the *Pieris* butterflies, *Pl. xylostella* moths prefer infested over uninfested leaves (Wilcoxon matched pair signed ranks test: Z = -4.541, N = 44, P < 0.001; Figure 5A). However, when phenidone was sprayed on the plants this eliminated the preference for the infested plants. The moths did not prefer infested plants over uninfested plants when they were sprayed with phenidone before infestation (Wilcoxon matched pair signed ranks test: Z = -1.542, N = 46, P = 0.123; Figure 5B), and did discriminate between infested plants sprayed with phenidone or Tween 20 solution, preferring the ones sprayed with Tween 20 (Wilcoxon matched pair signed ranks test: Z = -2.892, N = 40, P = 0.004; Figure 5C).

Plutella xylostella did not discriminate between the systemic leaves

(undamaged leaves from infested plants) from infested and uninfested plants, although they tended to prefer the leaves from infested plants (Wilcoxon matched pair signed ranks test: Z = -1.635, N = 41, P = 0.102). Not surprisingly, the moths did also not discriminate between leaves from uninfested and infested plants when both were treated with phenidone, but the tendency we observed in the previous comparison disappeared (Wilcoxon matched pair signed ranks test: Z = -0.110, N = 44, P = 0.912).

The moths also deposited many eggs on the plastic cages. The distribution of eggs that were deposited on leaves or on the cage differed per treatment (contingency table test: $\chi^2 = 130.3$, df = 4, P < 0.001). The percentages seem to depend on the attractiveness of the leaves offered. When the leaves from the most attractive plants in our tests (locally damaged plants without phenidone treatment) were offered as one of the two alternatives, the moths deposited on average 36 and 46% of their eggs on the cage, while for the other tests the percentages varied from 52 to 59%.

OPDA analysis

To test whether phenidone treatment of Brussels sprouts plants affects the accumulation of octadecanoid-pathway intermediates downstream from LOX, we analysed OPDA levels. Application of phenidone before infestation resulted in a lower concentration of OPDA compared to the infested plant without phenidone for both herbivores (Mann-Whitney U test, *P. rapae*: Z = -2.626, N = 8, P = 0.009, *P. brassicae*: Z = -1.995, N = 8, P = 0.046; Figure 6).

Volatile analysis

In the headspace of phenidone-treated intact plants, *P. brassicae*-infested plants, and phenidone-treated *P. brassicae*-infested Brussels sprouts plants, we detected 18 compounds (alcohols, esters, aldehydes, and terpenoids) (Table 1). Major compounds in all volatile blends were sabinene, limonene, (*Z*)-3-hexen-1-yl acetate and 1,8-cineole. Plants with feeding damage emitted many compounds in larger amounts than intact plants. Contrary to what was expected, green leaf volatile and terpene emission was not clearly inhibited by phenidone treatment. The PCA extracted one significant principal component that explained 44.7% of the variation in the data (Figure 7A). Although all three treatments showed considerable variation, they could be significantly separated by PLS-DA (1 PLS-component, $R^2X = 0.418$, $R^2Y = 0.333$, $Q^2 = 0.164$). PLS-DA mostly separated the infested plants from phenidone-treated intact plants (PHEN), while the phenidone-treated infested plants (PHEN+PB) differ slightly from the Tween-treated infested plants (PB) (Figure 7B). Compounds that were most influential for the separation of the groups (based on VIP-values) were (*Z*)-3-hexen-1-yl acetate (VIP = 1.49), (*Z*)-

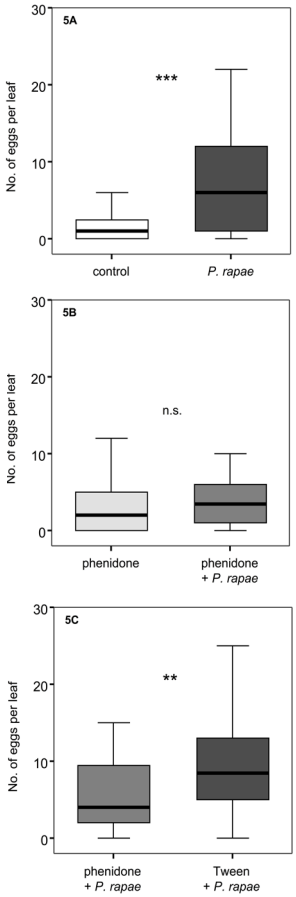


Figure 5. Oviposition preference of *Plutella xylostella* on A) *Pieris rapae*-infested vs. uninfested (control) leaves, B) *P. rapae*-infested leaves with or without phenidone C) phenidone-treated leaves with or without *P. rapae*. The thick line indicates the median, the box represents the interquartile range from first to third quartile (Wilcoxon matched pair signed rank test, *** P < 0.001, ** P < 0.01, n.s. not significant).

3-hexen-1-ol (VIP = 1.46) and (*E*)-DMNT (VIP = 1.42)(Figure 7C).

Discussion

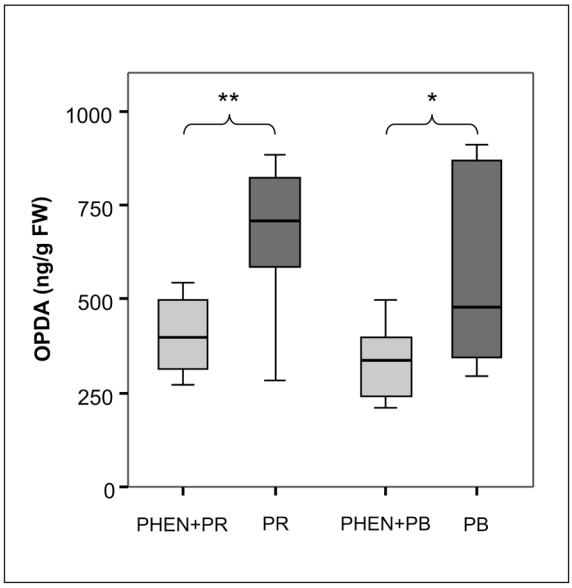


Figure 6. Effect of phenidone treatment and caterpillar infestation on OPDA (12-oxo-phytodienoic acid) levels in *Pieris rapae*-infested (PR), *P. brassicae*-infested (PB), phenidone-treated *P. rapae*-infested (PHEN+PR) and phenidone-treated *P. brassicae*-infested (PHEN+PB) Brussels sprouts plants. The thick line indicates the median, the box represents the interquartile range from first to third quartile; ** P < 0.01, * P < 0.05.

We demonstrate that the inhibition of the first enzymatic step in the octadecanoid pathway influences the responses of three herbivores and a parasitoid towards infested *Brassica* plants. To our knowledge this is the first study that uses inhibitors of the octadecanoid pathway, such as phenidone, to study not only plant responses, but to investigate also the effect of LOX inhibition on the behavioural responses to the plants by insects at two trophic levels. We show that treatment with phenidone before infestation reduces lipoxygenase-dependent plant responses that subsequently influence the behavioural responses of herbivorous and carnivorous insects. This approach provides insight into the sensitivity of insects to the induction of the octadecanoid pathway by reducing induction by caterpillars. In this way, it is possible to include the visual cues caused by feeding damage while eliminating chemical cues. Recently, an inhibitor of the MEP (methylerythritol 4-phosphate)-pathway, fosmidomycin, was used to study indirect defence in two tritrophic systems, one with the same species as also used in this study, i.e. Brussels sprouts, *P. brassicae* and *C. glomerata* and, in addition, a system consisting of Lima bean, the spider mite *Tetranychus urticae* and the predatory mite *Phytoseiulus persimilis* (Mumm et al., 2008). While in Brussels sprouts plants the fosmidomycin treatment did not have a strong effect on volatile production and no effect on oviposition behaviour of *P. brassicae* or attraction of *C. glomerata*, in Lima bean plants fosmidomycin completely inhibited the emission of monoterpenes and TMTT and reduced the attractiveness of spider mite-induced Lima bean to predatory mites. These results elegantly elucidate the relative importance of terpenoids for predatory-mite attraction. Another study using an inhibitor of the shikimic pathway, glyphosate, showed that shikimic acid-induced plant volatiles were not influencing attraction of one parasitoid species *Co-*

tesia marginiventris, but were repellent for another, *Microplitis rufiventris* (D'Alessandro et al., 2006). Inhibitors of different pathways can therefore be successfully used to study induced indirect plant defences (D'Alessandro and Turlings, 2006; Bruinsma and Dicke, 2008).

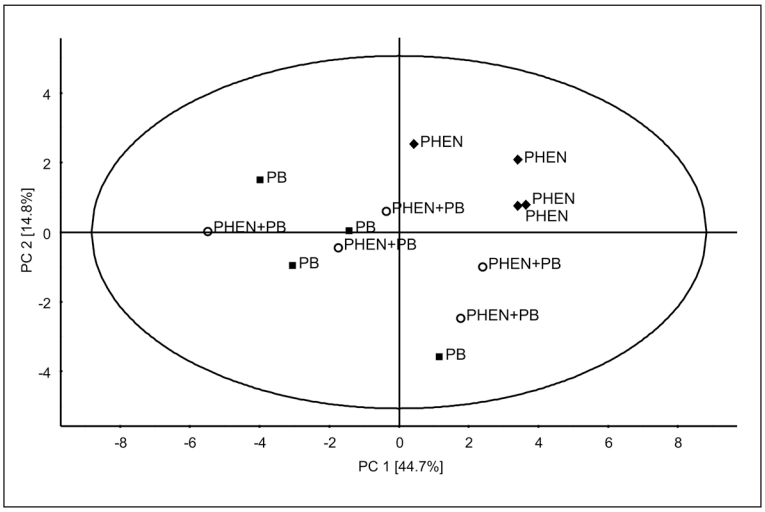
Phenidone and DIECA treatment of Brussels sprouts plants resulted in a reduced attractiveness of infested plants to *C. glomerata*. Although propyl gallate-treated plants also attracted fewer parasitoids, this difference was only marginally significant. Of the three inhibitors the LOX inhibitor phenidone caused the largest difference in the attraction of the parasitoid *C. glomerata*. Therefore, we performed all other experiments only with this inhibitor.

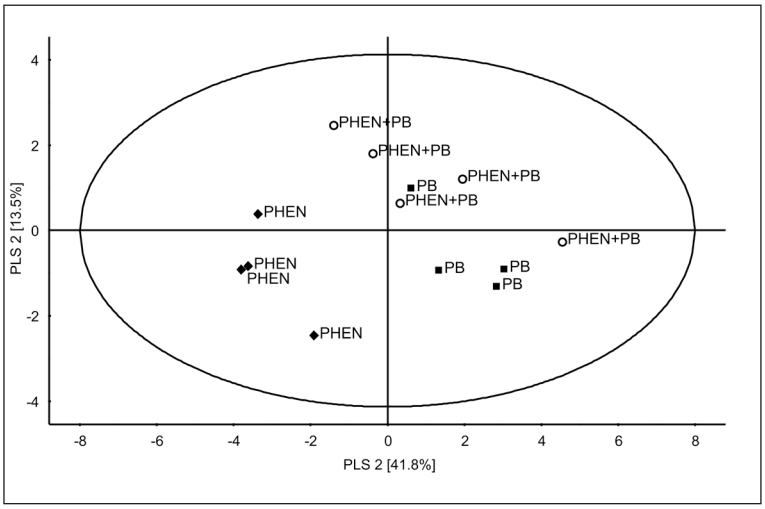
The herbivores *P. rapae* and *P. brassicae* were less sensitive to feeding induction and LOX inhibition respectively than their natural enemy *C. glomerata*. We found that *P. rapae* does not even discriminate between undamaged plants and plants with feeding damage from 15 caterpillars for 24 hours. Only high densities of *P. rapae* caterpillars (45 per plant) that are much higher than densities occurring in the field (Poelman et al., in prep), or long term damage (one week) changes the oviposition preference of the butterflies. Previous studies have obtained diverse results with *P. rapae* oviposition. Poelman et al. (in prep) found no

	Compound	*Pieris brassicae*	Phenidone + *P. brassicae*[a]	Phenidone[a]
1	2-methyl-1-propanol	1.3±1.0	n.d	3.3±1.8
2	hexanal	1.3±0.4	11.2±9.5	n.d.
3	(*Z*)-3-hexen-1-ol	6.5±1.9	6.6±1.7	0.5±0.3
4	α-thujene	18.1±4.6	15.0±3.5	9.8±1.7
5	α-pinene	10.9±2.0	9.3±1.6	6.6±1.0
6	benzaldehyde	26.5±2.7	26.5±3.9	19.9±6.1
7	sabinene	81.0±13.5	72.3±16.7	45.4±7.5
8	β-pinene	7.6±1.9	5.2±1.7	4.2±1.2
9	myrcene	16.5±5.5	16.9±4.6	10.4±2.1
10	(*Z*)-3-hexen-1-yl acetate	29.6±14.0	27.8±7.1	1.0±1.0
11	hexyl acetate	1.0±1.0	0.9±0.6	n.d.
12	limonene	44.0±9.6	48.1±11.3	32.0±6.5
13	β-phellandrene	0.4±0.2	0.6±0.3	0.3±0.2
14	1,8-cineole	29.3±6.2	25.7±7.4	14.4±3.9
15	β-isophorone	20.±1.2	3.3±1.5	3.6±1.2
16	(*E*)-4-thujanol	3.6±2.6	1.7±0.7	1.0±0.3
17	(*E*)-4,8-dimethyl-1,3,7-nonatriene[b]	1.3±0.4	0.5±0.3	n.d.
18	isophorone	1.8±1.4	2.2±1.4	1.3±0.9
19	2-tertiary-butylcyclohexyl acetate	0.5±0.5	n.d	0.7±0.4
20	α-gurjunene	3.3±1.3	2.7±1.0	4.2±1.5
	Total	358.8±46.4	331.6±74.3	200.5±40.2

[a] n.d.: not detected
[b] (*E*)-DMNT

Table 1. Volatile compounds detected in the headspace of Brussels sprouts plants treated with 2 mM phenidone with 0.1% Tween 20 (N = 4), infested with *Pieris brassicae* and sprayed with phenidone with Tween 20 (N = 5) or infested with *P. brassicae* and sprayed with Tween 20 (N = 4) 24 hours before headspace collection. Mean (± SE) of GC peak area (units/g fresh weight).





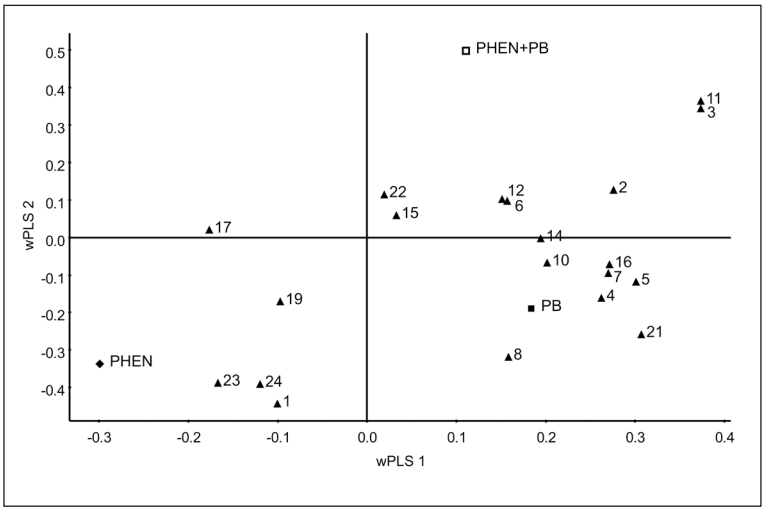


Figure 7. Multivariate data analysis of the volatile pattern of plants infested with *Pieris brassicae* (PB), phenidone-treated *Pieris brassicae*-infested plants (PHEN+PB), and phenidone-treated intact plants (PHEN). Percentage variation explained between brackets. The ellipse defines the Hotelling's T^2 confidence region (95 %). (A) Score plot of PCA, and (B) score plot of PLS-DA and (C) loading plot of PLS-DA as based on the relative amounts of 20 volatile compounds from the differently treated Brussels sprouts plants. Compound numbers correspond to numbers in Table 1.

preferences of *P. rapae* for either infested or uninfested leaves of two cabbage cultivars when infested with 10 *P. rapae* for one week. In contrast, Sato et al. (1999) observed a preference for *Rorippa indica* plants infested with 100 *P. rapae* larvae for 24 h in a field experiment; however, this experiment tested only one plant per treatment and the caterpillar dose was unrealistically high. In Chapter 3 (Bruinsma et al., 2007) I compared the oviposition preference of *P. rapae* on JA-induced and non-induced leaves and found that they preferred non-induced leaves, at doses of 0.1 and 1 mM JA, but did not discriminate at lower doses, whereas the parasitoid *C. glomerata* is already attracted by plants induced with 0.01 mM (Chapter 4). Thus, both after induction with JA or herbivores, and inhibition of the octadecanoid pathway with phenidone *C. glomerata* is more sensitive to changes in plant chemistry than *P. rapae*.

Pieris brassicae was more selective than *P. rapae*. Large cabbage white butterflies discriminated between uninfested plants and plants that were damaged by 15 caterpillars for 24 h. Treatment with phenidone of both uninfested and infested plants eliminated this preference. Since *C. glomerata* was less attracted to plants treated with phenidone, lower induction levels due to phenidone treatment may signal a lower risk of parasitism for *P. brassicae*, and therefore reduce the benefit of discrimination. Possibly the different oviposition strategies of the two species can explain the difference in selectiveness between *P. rapae* and *P. brassicae*. *Pieris rapae* is a solitary butterfly, which means that it lays a single egg at a time and spreads its eggs over many plants. *Pieris brassicae*, on the other hand, is a gregarious butterfly and lays its eggs in clusters of about 20–100 eggs (Davies and Gilbert, 1985). Therefore the choice of an oviposition site has higher fitness consequences for *P. brassicae* than *P. rapae*; *P. brassicae* can therefore be expected to be more selective.

Our results show that blocking LOX activity reduces the plant's indirect defence. In the arms race between plants and herbivores, any herbivore that would be able to silence a plant's induced defence signalling would have a higher chance to survive and reproduce. One way of accomplishing this might be to block LOX activity. No herbivore has yet been shown to repress LOX activity. However, caterpillar saliva has shown to be able to counteract nicotine production in response to wounding in tobacco (Musser et al., 2002; Musser et al., 2005). Furthermore, for spider mites, intraspecific variation exists in traits regarding susceptibility to JA-dependent defences as well as repression of these defences in their host plant (Kant et al., 2008), but the mechanisms underlying this observed repression have not yet been elucidated.

In contrast, *Pl. xylostella* prefers to oviposit on plants infested with *P. rapae* (Figure 5; Poelman et al., in prep; Shiojiri et al., 2002). Shiojiri et al. (2002) showed that this is a beneficial strategy for *Pl. xylostella*, since its host *Cotesia plutellae* was less efficient in host searching on plants infested with both *P. rapae* and *Pl. xylostella*, than on plants with only *Pl. xylostella*, which resulted in lower parasitism rates on plants with *P.*

rapae. We show that the preference of *Pl. xylostella* for *P. rapae*-infested plants over uninfested plants is LOX-dependent because phenidone treatment of uninfested and infested plants eliminated the preference. Moreover, *Pl. xylostella* females preferred infested plants sprayed with Tween over infested plants sprayed with phenidone, indicating that the phenidone treatment can reduce the induction of oviposition cues for *Pl. xylostella*. *Plutella xylostella* did not show the same preference for the systemic leaves (undamaged leaves from infested plants) as for the locally damaged leaves. Possibly the induction of systemic leaves does not reach sufficiently high levels of defence compounds or it requires more than 24 h induction. *BoLOX* (a lipoxygenase gene that is involved in the defence response of Brussels sprouts plants) expression after 24 h of feeding by 16 *P. rapae* caterpillars was upregulated in both local and systemic leaves of Brussels sprouts, but approximately 40-fold higher in local than in systemic leaves (Zheng et al., 2007). Probably the level of induction of systemic leaves is not sufficient (yet) for *Pl. xylostella* to prefer induced systemic leaves over non-induced systemic ones for oviposition.

Phenidone reduced OPDA accumulation upon *Pieris* feeding (Figure 6). Since phenidone inhibits LOX, an early step of the octadecanoid pathway, and we show that it reduces OPDA accumulation in response to herbivory, we expected that phenidone treatment would reduce emission of green leaf volatiles and terpenoids; which were shown to be induced by JA treatment of Brussels sprouts plants in Chapter 4 and 6. The volatile emission differed in many compounds between intact and infested plants; however, phenidone treatment only slightly changed volatile emission of infested plants (Table 1 and Figure 7). Possibly, the large variation of volatile emission combined with low sample size obscured detection of subtle changes and changes in minor compounds that may be important for the associated insects. To test the effect of phenidone itself we applied phenidone to intact leaves and compared preference of the herbivores for Tween 20- or phenidone-treated leaves. For both *P. brassicae* and *Pl. xylostella* phenidone did not affect oviposition preference. Furthermore, OPDA levels confirmed the inhibition of the plants defence response. Therefore, we ascribe our results to inhibition of (part of) the defence response of the plant rather than to odours from phenidone itself.

The results of the insect bioassays, as well as the OPDA and volatile emission analyses showed that phenidone does not block induction completely. The lack of complete inhibition could be due to the reduction of only a fraction of LOX enzymes to the inactive form by phenidone, or to induction of plant defences by alternative routes when LOX activity is blocked. Therefore, we cannot pose that LOX activity is crucial, but we do show that it plays an important role in plant defence against herbivorous insects in Brussels sprouts plants. The results from this study comply with those reported in Chapter 3 en 4 in this thesis, in which we show that JA treatment of Brussels sprouts plants mediates

direct and indirect defence.

The role of lipoxygenase in direct and indirect defences against herbivorous arthropods has now been demonstrated in several ways, through genotypic and phenotypic induction and inhibition. Genetic inhibition of LOX in several plants species, such as *Arabidopsis thaliana*, *Nicotiana attenuata* and potato, resulted in reduced volatile emission, defence gene expression, attraction of parasitoids and increased plant damage in the field (e.g. Royo et al., 1999; Van Poecke and Dicke, 2002; Van Poecke et al., 2002; Kessler et al., 2004). A difference between LOX inhibition with chemicals and genotypic modification is that phenidone blocks all LOXs, whereas genetic modification can block expression of one specific *LOX* gene. Combining different approaches, phenotypic manipulation (elicitors and inhibitors of different pathways) and genotypic differences (using mutants and genetically modified plants), and studying plant genomics, metabolomics as well as insect behaviour and interactions, will help us to increase our understanding of the infochemical network that mediates interactions between plants, herbivores and their natural enemies.

Acknowledgements
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BEELD GROEP

Chapter 8

Summarising discussion: induced plant defence in a community context

Maaike Bruinsma

Infochemical use in insect–plant interactions

Chemical communication plays an important role in the interactions between plants and insects. When an herbivore is feeding on a plant, the plant can respond with the production of specific volatiles and toxins (Karban and Baldwin, 1997). Herbivorous insects can use the chemical changes as information on the infestation status of the plant, determining the suitability of the host plant for feeding or oviposition; infestation may affect herbivore development on a plant, competition for food and risk of predation or parasitism, because of attraction of natural enemies (Figure 1). Carnivorous insects can use this information to find their host or prey, which may be small and inconspicuous, while the plant emits cues that are less reliable, but easier to detect (Vet and Dicke, 1992). Infochemicals can therefore have an important influence on insect–plant interactions. Studying how infochemicals are involved in these interactions is a major challenge, that requires a multidisciplinary approach (Baldwin et al., 2002; Dicke et al., 2004).

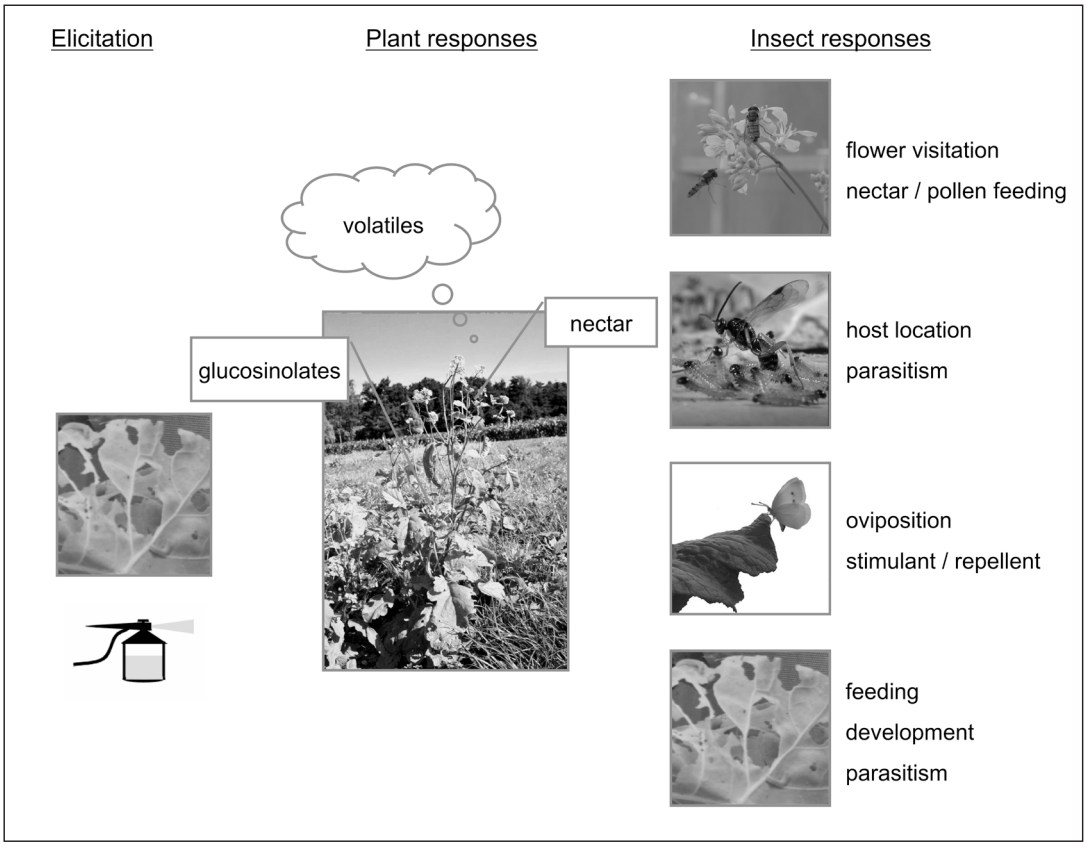

Figure 1. Multiple effects of induced plant responses on insect–plant interactions. Induced plant responses can have positive (host location, oviposition stimulant, sequestration) or negative (repellent, toxic) effects on the insect community associated with the plant.

Phenotypic manipulation

This project was part of a large NWO-VICI-project in which two PhDs and two post-docs were studying the role of infochemicals in insect–plant interactions using an ecogenomic approach. In this project we used (1) a genetic approach by studying transgenic plants modified in signal transduction pathways and (2) a chemical manipulation approach to modify infochemical phenotype and studied if and how this affected a range of plant traits as well as the interactions with associated insects in field and laboratory studies. I took the phenotypic manipulation approach to study the infochemically-mediated interactions between plants and some selected members of the plant-related insect community (Chapter 1). The use of chemical elicitors and inhibitors of steps in the signal-transduction pathways leading to induced defence responses can help to gain insight into the importance of these specific steps in the induction of phenotypic changes (Chapter 2: Bruinsma and Dicke, 2008). Advantages of using elicitors and inhibitors, compared to herbivory, are that it allows to manipulate particular steps in pathways, as well as to induce plants in a dose-controlled manner, while with induction with herbivores the amount of damage inflicted to the plants is difficult to control. However, also with elicitors and inhibitors it is often difficult to link the applied dose to the strength of induction of the plant, as the plant may use alternative routes to express certain traits. Furthermore, as the signal-transduction pathways targeted by elicitors and inhibitors often have several end products, the manipulation can result in unwanted effects on other processes in the plant, such as flowering or senescence (Maciejewska et al., 2004; Wasternack, 2007). Therefore, experiments using elicitors or inhibitors should preferably use rather short incubation times (hours to days), to avoid developmental differences due to treatment (Mumm et al., 2008). Although genetically modified plants have the disadvantage to be deficient in a certain trait during development, the advantage is that a specific step can be blocked completely. Combining data obtained from genotypic and phenotypic manipulation experiments is therefore the most comprehensive approach available.

Jasmonic acid application does not fully mimic herbivory: possibilities and limitations of phenotypic manipulation

The manipulation of odour blends of Lima bean plants has led to insight into the role of several induced volatile infochemicals in the attraction of predatory mites to spider mite-infested plants. It was shown that treatment of plants with jasmonic acid (JA) increased the attractiveness to predatory mites compared to untreated plants and induced a volatile blend that was similar to, but not the same as, the blend emitted after spider mite infestation (Dicke et al., 1999). Spider-mite infested plants were more attractive than JA-treated plants. The addition of methyl salicylate (MeSA), that was not induced by JA application, to the odour blend of JA-treated Lima bean plants, rendered the plants even more attractive to the predatory mites (De Boer and Dicke, 2004). Recently, the relative importance of terpenoids for predatory mites was shown

using an inhibitor of a specific step in the terpenoid biosynthesis pathway (Mumm et al., 2008).

Jasmonic acid is often used as a mimic of induction by herbivory. However, my results also indicate that the JA-induced volatile emission differs from herbivore-induced volatile emission (Chapter 4), and more nectar is secreted in flowers from herbivore-infested plants than in flowers from JA-induced plants (Chapter 6). The behavioural responses of herbivores and parasitoids differed in strength between JA- and herbivore-induced plants, but compared to non-induced plants, both treatments were favoured by parasitoids (Chapter 4), while *Pieris* butterflies avoided oviposition on these plants (Chapter 3). The results indicate that JA-mediated responses do play an important role in plant defence against herbivorous insects, and can be used to induce defence responses in many plant species; however, one should be careful with comparing JA induction with herbivore infestation. This was also shown at the transcriptional level. In a study on *Arabidopsis thaliana*, infestation with each of two herbivorous insects and two microbes induced JA in the plants, but global transcriptional profiling showed that only 32-69% of the JA-responsive genes were actually upregulated by the four plant attackers (De Vos et al., 2005).

Induced defence in a multitrophic context

I studied the effects of induction and inhibition of induced defence signal-transduction pathways on volatile emission, glucosinolate contents, oxylipin accumulation and nectar secretion. To quantify the level of induced defences as well as the effects on mutualistic organisms, I also studied the responses of herbivores, parasitoids and pollinators to differently induced plants, and discuss these behavioural responses in relation to the changes in the plant traits that were quantified. I summarize and discuss the results per trophic level.

Early events in plant defence responses

Upon insect damage several processes take place within the plant, within different time windows. Early events in response to attack are changes in plasma transmembrane potential (within seconds to minutes) (Maffei et al., 2007a). When *Spodoptera littoralis* feeds on Lima bean plants membrane depolarisation is followed by a flow of Ca^{2+} from the apoplast or organelles or both into the cytoplasm (Maffei et al 2006). Signal molecules such as H_2O_2, a strongly depolarizing molecule that can be induced or introduced by the feeding insect, affect plasma transmembrane potential and are important for early recognition of herbivore attack. As long as the herbivores feed on the plant the H_2O_2 levels increase. The network of interactions between phytohormones regulates the fine-tuned attacker-specific response in a later stage. However, the connection between early perception and later metabolic changes is not yet well understood. Phytohormones such as jasmonic

acid (JA), salicylic acid (SA) and ethylene (ET) are detectable within minutes (to hours) after damaging the plant. Gene expression and resulting metabolic changes follow later, from several minutes to days after the attack.

Alamethicin (ALA) is an ion channel-forming peptide mixture from the fungus *Trichoderma viride*. Influencing ion fluxes can induce early events in the defence response and resulted in activation of JA and SA in Lima bean plants, probably preceded by an abundant generation of reactive oxygen species such as H_2O_2 (Engelberth et al., 2001; Maffei et al., 2007b). ALA treatment of Lima bean resulted in the emission of several volatile compounds that are attractive to predatory mites. In Brussels sprouts a quantitative increase in volatile emission was observed, rather than a qualitative response such as occurs in Lima bean, but it was also demonstrated that parasitoids are attracted to Brussels sprouts plants after application of ALA (Chapter 6). It would be interesting to measure phytohormone levels in response to ALA treatment of Brussels sprouts plants to elucidate which signal-transduction pathways are induced by ALA application in this plant species.

The octadecanoid pathway

Most phenotypic manipulations of plant defence described in this thesis acted on the octadecanoid signal-transduction pathway (Figure 2). Jasmonic acid (JA) is a key hormone in this pathway and involved in direct as well as indirect plant defences against herbivores. This phytohormone increases volatile emission, toxin levels and upregulates defence gene expression. These changes in plant traits affect members of the insect community associated with the plants and result in higher parasitism rates of herbivores, attraction of predators, and reduced oviposition and development of herbivores (e.g. Chapter 3-5; Baldwin, 1998; Dicke et al., 1999; Thaler, 1999a; Thaler et al., 2002a).

JA biosynthesis is suggested to be regulated by positive feedback, as JA can induce all genes encoding enzymes involved in JA biosynthesis (Wasternack, 2007). This fits well with the observation in Brussels sprouts that *BoLOX* (a lipoxygenase gene from *Brassica oleracea*) is induced by JA application (Zheng et al., 2007). *BoLOX* expression is also upregulated by wounding and feeding by a range of herbivores, including *Pieris rapae* and *P. brassicae* (Zheng et al., 2007) that I also used for induction in several chapters in this thesis. Inhibition of LOX, either through the application of an inhibitor or through genetic modification, decreases volatile emission, extrafloral nectar secretion and attraction of parasitoids, and changes herbivore oviposition preference (Chapter 7; Koch et al., 1999; Heil et al., 2001; Shiojiri et al., 2006b). *LOX* expression upon herbivore infestation reaches a maximum level within 24 hours in Brussels sprouts plants (Zheng et al., 2007). Interestingly, *BoLOX* expression does not increase when caterpillar density increases from 2 to 32 per plant (Zheng et al., 2007). However, the degree of attraction of parasitoids increases concomitantly with increasing infestation rate from

1 to 90 caterpillars (Geervliet et al., 1998). In *A. thaliana* induction of JA biosynthesis takes place upon damage, despite the presence of abundant LOX, AOS and AOC proteins (Stenzel et al., 2003). These results suggest that not LOX, but the amount of substrate is rate limiting (Wasternack, 2007; Zheng et al., 2007). This concurs with the observation that addition of linolenic acid can increase volatile emission and proteinase inhibitor levels in Lima bean plants and tomato, respectively (Farmer and Ryan, 1992; Koch et al., 1999).

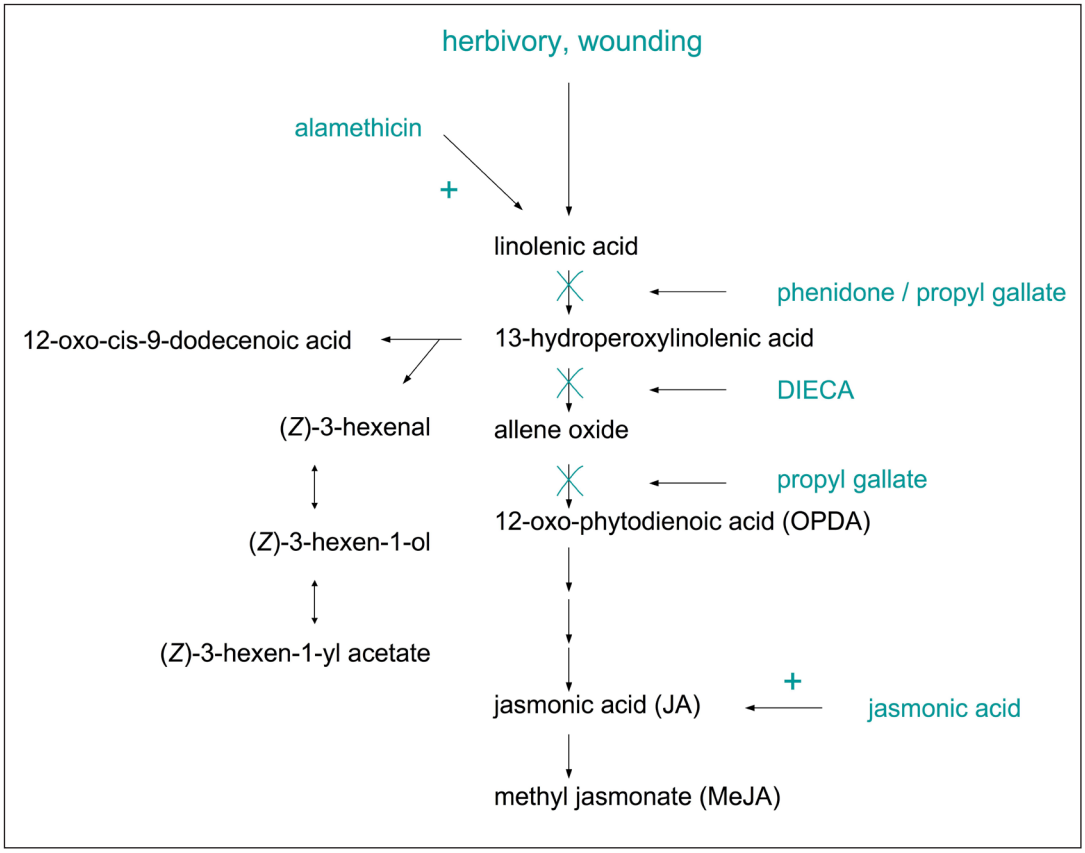

Figure 2. Representation of the pathway of jasmonic acid biosynthesis from α-linolenic acid. Different elicitors and inhibitors are indicated. Jasmonic acid is suggested to be regulated by positive feedback, therefore JA application also affects steps upstream of JA and can induce green leaf volatiles such as (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate.

The expression of many defence related genes is controlled by a family of mediators known as jasmonates (Gfeller and Farmer, 2004). Using an *A. thaliana* mutant it was demonstrated that not only JA, but also another jasmonate family member upstream of JA, OPDA (12-oxo-phytodienoic acid), can mediate plant resistance in the absence of JA (Stintzi et al., 2001). The JA-deficient plants were resistant against a fungal pathogen and an insect herbivore. This implies that OPDA plays a role in defence signalling. It was suggested that OPDA, possibly in combination with dinor-OPDA, fine-tunes the defence response in concert with JA (Stintzi et al., 2001). I measured the accumulation of the octadecanoid-pathway intermediate OPDA to address the induction and inhibition of the octadecanoid pathway in Brussels sprouts after herbivore infestation and inhibitor application. In Brussels sprouts, feeding by the herbivores

Pieris rapae and *P. brassicae* resulted in higher levels of OPDA in the leaves. A larger number of *P. rapae* caterpillars feeding on the plant increased OPDA levels even more (results not shown). Herbivore-infested plants treated with the inhibitor phenidone had much lower OPDA levels, which were only slightly above the levels recorded in non-induced plants (Chapter 7).

In most plants, damage results in a transient burst of JA. Upon mechanical wounding or application of regurgitant, JA levels peak within a few minutes to several hours (e.g. Reymond et al., 2000; Ziegler et al., 2001). After a single wounding event JA levels rapidly level off to the concentration present before wounding. In case of herbivore feeding, JA levels increase for several hours (Ziegler et al., 2001; Reymond et al., 2004) and JA-induced phytochemicals can slow down feeding of larvae. In turn, reduced feeding because of unfavourable plant chemistry or inherent to diurnal rhythmicity results in lower JA levels, and after larvae resume feeding, JA levels can rapidly increase again (Ziegler et al., 2001). In *A. thaliana* OPDA accumulation in response to mechanical wounding was slower than JA accumulation (Reymond et al., 2000; Stintzi et al., 2001). In systemic leaves (undamaged leaves adjacent to damaged leaves) of Lima bean plants only OPDA accumulation was found, and no increase in levels of JA (Schulze et al., 2007), suggesting that OPDA might be relevant for induction of a certain subset of defence responses (Maffei et al., 2007a).

Fine-tuning the defence response

The octadecanoid pathway is not the only pathway involved in plant defence responses. Salicylic acid and ethylene are the major phytohormones also involved in herbivore-induced plant defence (Dicke and Van Poecke, 2002; Van Poecke, 2007). As suggested in several chapters in this thesis, other phytohormones play an important role in fine-tuning the defence response. It is usually thought that the octadecanoid pathway plays an important role in the protection of plants against herbivorous arthropods, whereas the salicylate pathway is mainly involved in the protection against pathogens (Karban and Baldwin, 1997; Dempsey et al., 1999; Felton and Korth, 2000; Dicke and Van Poecke, 2002; Van Poecke, 2007). These pathways can negatively interact with each other (e.g. Doares et al., 1995; Niki et al., 1998; Thaler et al., 2002c; Cipollini et al., 2004), but not necessarily so, depending on induction and the response type measured (Niki et al., 1998; Schenk et al., 2000; Thaler et al., 2002c). Furthermore, even negative interactions between these two signal pathways do not always decrease plant resistance to herbivores or pathogens (Shimoda et al., 2002; Thaler et al., 2002c). Also ethylene has been shown to interact with JA-inducible defence responses (Kahl et al., 2000; Stotz et al., 2000; Horiuchi et al., 2001).

This network of signalling, induced by different stressors, finally results in different plant phenotypes to which the associated insect community

is exposed and, thus, can respond. In three chapters in this thesis volatile emission in response to diverse treatments is described (Chapters 4, 6 and 7). In all studies the major volatiles emitted from Brussels sprouts were sabinene, limonene, 1,8-cineole and (*Z*)-3-hexen-1-yl acetate. Herbivore feeding and elicitor treatment increased volatile emission. However, as the three studies employed different experimental treatments, the volatile blends emitted are not directly comparable. A striking feature was the variability of the volatile blends that occurred despite strictly controlled experimental conditions.

Comparison of volatile emission within one study allows assessment of the effect of different elicitors. In Chapter 4, JA treatment was compared to herbivore infestation. Brussels sprouts plants were shown to emit larger amounts of volatiles after application of 1 mM JA than after feeding by 30 *Pieris rapae* caterpillars, but were shown to be less attractive to parasitoids. After application of ALA to Brussels sprouts plants considerable variation in volatile emission was observed, but also a consistent attraction of parasitoids (Chapter 6). Although at the doses used, ALA treatment resulted in a lower total amount of volatiles, ALA-treated plants were as attractive to the parasitoids as JA-treated plants that emitted much higher quantities of volatiles. Differences in plant volatile emission after induction by JA, ALA or herbivore feeding, stresses the importance of cross-talk between the signalling pathways involved in volatile production.

Responses of herbivores to induced defences

I studied three specialist herbivore species that feed on Brassicaceous plants, *Pieris rapae*, the small cabbage white, *P. brassicae*, the large cabbage white, and *Pl. xylostella*, the diamondback moth. The three species were shown to differ in their oviposition preferences. *Pieris brassicae* showed the strongest avoidance of infested plants (Chapter 7), as well as JA-induced plants (Chapter 3). *Pieris rapae* did not discriminate between plants with a low level of infestation (15 caterpillars per plant), but did so after damage had increased, either through longer term infestation or increased densities (Chapter 7). Also JA-induction of plants was shown to cause oviposition preference for non-induced plants in *P. rapae* (Chapter 3). Development of *P. rapae* caterpillars was slower on JA-induced plants. Oviposition preference for non-induced plants is therefore adaptive, since it sustains faster development of offspring and will reduce the parasitism risk. Also on *P. rapae*-induced cabbage plants the development rate of *P. rapae* was reduced (Poelman et al., unpublished results). These results demonstrate that also specialist herbivores can be negatively affected by induced defences.

Contrary to *P. brassicae*, *Pl. xylostella* oviposits preferentially on plants previously infested with *P. rapae* compared to uninfested plants. A study by Shiojiri et al. (2002) showed that this can be a beneficial strategy for *Pl. xylostella* since parasitism rates by its parasitoid *Cotesia vestalis* (= *plutellae*) were lower on plants infested with heterospecific caterpillars.

Plant treatment with an inhibitor of lipoxygenase before infestation reduced oviposition preference of *Pl. xylostella* for infested over uninfested plants (Chapter 7), suggesting that the oviposition preference is based on chemical cues and not on visual cues. Also, oviposition preference of *P. brassicae* for undamaged plants was less pronounced after application of a lipoxygenase inhibitor (Chapter 7).

Glucosinolates, secondary metabolites in Brassicaceous plants that can be used by specialist herbivores as oviposition cues, did not explain the oviposition preference of *P. brassicae* and *P. rapae* for non-induced leaves over JA-induced leaves. While application of a single compound or a blend of glucosinolates to an inert substrate induced oviposition in *P. rapae*, the application of glucosinolate blends, in a composition and quantity such as they occurred in induced and non-induced plants, on this inert substrate did not cause the same discrimination as observed for the leaves (Chapter 3). This corresponds to recent data by Van Leur et al. (2008) on oviposition preference of small cabbage white butterflies. Van Leur et al. (2008) studied the oviposition of *P. rapae* on two distinct chemotypes of *Barbarea vulgaris*, one of which produces mainly glucobarbarin and the other chemotype mainly gluconasturtiin. The butterflies oviposited on both chemotypes, and despite the large difference in glucosinolate content the butterflies did not discriminate between the two chemotypes. These results indicate that *Pieris* butterflies do indeed use glucosinolates as oviposition cues but do not necessarily discriminate between specific profiles.

Parasitoid attraction to induced plants

The parasitoid species studied most extensively in this thesis, *Cotesia glomerata*, was more sensitive in its response to changes in plant defence than its herbivorous hosts *P. brassicae* and *P. rapae*. Both in the induction (Chapter 4) and in the inhibition (Chapter 7) of plant defences, the parasitoids responded more strongly to inhibitor treatment or to treatment with a lower elicitor dose. However, herbivores and parasitoids are likely to use different cues that may be differentially affected by the treatments. *Pieris* butterflies respond more strongly to non-volatile glucosinolates, whereas volatiles are thought to be major cues in parasitoid host location behaviour. Parasitoids of Lepidopteran larvae display antennal responses to a wide range of compounds, including alcohols, aldehydes, esters and terpenoids (Smid et al., 2002; Gouinguené et al., 2005). Green leaf volatiles, such as (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and hexanal, evoke most consistent and sensitive antennal responses in three parasitoids of Lepidopteran larvae, *Cotesia marginiventris*, *Campoletis sonorensis* and *Microplitis rufiventris* (Gouinguené et al., 2005). Plant compounds that are emitted in larger amounts from induced plants in many plant species are green leaf volatiles, such as (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, and terpenoids, such as (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and the benzenoid methyl salicylate (MeSA), and have been suggested to be important for host location behaviour of several parasitoid wasps (e.g.

Reddy et al., 2002; Ibrahim et al., 2005; Scascighini et al., 2005; Shiojiri et al., 2006b; Pinto et al., 2007; Wei et al., 2007).

The fatty acid derivative (*Z*)-3-hexen-1-ol is an evolutionarily conserved and ubiquitous compound and emitted by almost all plant species immediately upon damage (Wei et al., 2007). Possibly (*Z*)-3-hexen-1-ol is an important cue for parasitoids, and other specifically induced compounds may improve precision of host selection. Innate responses to more general signals can be advantageous. In a heterogenous environment it may be beneficial to innately respond to general cues, until more specific cues are learned through experience (Vet et al., 1998). Generalists are expected to be better learners than specialists, since their host range may vary more per generation (Vet et al., 1998; Steidle and van Loon, 2003). While *Cotesia glomerata* indeed responds to the plants soon after induction by caterpillar feeding, which results in the emission of increased amounts of (*Z*)-3-hexen-1-ol in combination with 1,8-cineole, (*Z*)-3-hexenyl acetate and benzylcyanide (Scascighini et al., 2005), another study on *C. glomerata* demonstrated that (*Z*)-3-hexenol was not attractive when offered in combination with an intact plant, whereas (*Z*)-3-hexenyl acetate and (*E*)-2-hexenal did increase attractiveness to the parasitoids (Shiojiri et al., 2006b).

While induced volatile blends can be highly variable between plant treatments and plant species, they can evoke similar responses of the parasitoids. JA treatment of Brussels sprouts (Chapter 4) and black mustard plants (Chapter 5) resulted in a similar behavioural response in *Cotesia glomerata* parasitoids, while volatile emission differs considerably between the two species (Bukovinszky et al., 2005). Although it is still unclear which compounds are responsible for the attraction of most parasitoids studied, it is clear that, above a certain threshold, it is not only quantity that determines attractiveness. JA-induced plants emitted more volatiles than herbivore-infested plants, but were less attractive (Chapter 4), and in Chapter 6 it is shown that alamethicin induces lower release rates of volatiles than JA, but is as attractive to parasitoids. Previously, a similar pattern was observed for two plant species, Brussels sprouts and white mustard. Whereas white mustard emits lower amounts of volatiles *D. semiclausum* parasitoids are more attracted to these plants than to Brussels sprouts (Bukovinszky et al., 2005).

It has also been suggested that non-attractive compounds can mask the attractiveness to parasitoids (D'Alessandro et al., 2006). For example, root herbivory induces an increase in aboveground volatile emission of sulphurous compounds as well as low levels of attractants, but overall reduces the attraction of aboveground parasitoids (Soler et al., 2007). In Chapter 4 parasitoids were more attracted to herbivore-infested plants than to JA-treated ones that emitted larger amounts of volatiles; possibly some compounds in the volatile blend were present in amounts that became repellent to the parasitoids or masked other attractive volatile compounds.

Trade-off between defence and pollination?

Since JA induction changes many plant defence traits such as volatile emission, glucosinolate levels, OPDA levels and *BoLOX* gene expression, it is interesting to see whether these phenotypic changes of the plant also affect other associated insects like pollinators. Shorter but more frequent visits by pollinators can be beneficial for pollination of the plant, and may optimize pollen transfer (Kessler and Baldwin, 2007). Pollinators respond to a range of plant cues including volatile emission, nectar secretion and flower colour and number. Induction of defence responses with JA in flowering mustard plants reduced nectar quantity compared to control and herbivore-infested plants and could therefore be expected to affect pollinator visitation (Chapter 5). Nectar sugar concentrations did not change after JA-application. Pollinators did not show a change in behaviour towards JA-induced plants, in contrast to parasitoids and herbivores, that reacted differently to induced black mustard plants than to control plants (Chapter 5). Pollinator visitation can be influenced by herbivore feeding, both above- and belowground (Strauss et al., 1996; Adler et al., 2001; Poveda et al., 2003;2005). However, the mechanisms behind this are not yet known (Kessler and Halitschke, 2007). In one study the change in flower number could explain the difference in behaviour of honeybees; however, for syrphid fly attraction to wild radish this could not explain the change in flower visitation (Lehtilä and Strauss, 1997).

Future perspectives

Over the last decade the knowledge on induced defence responses has increased enormously. However, most studies have focused on vegetative plants in laboratory situations and induction with just a single herbivore. Advances are therefore to be made by (1) investigating infestations by multiple herbivore species (2) studying flowering plants, (3) performing observations and experiments in the field, and (4) using a multidisciplinary approach, building on the advances made in molecular, chemical and ecological reasearch. In the field, plants are commonly attacked by more than one herbivore simultaneously or sequentially. Therefore, studies testing the effect of multiple infestation and the effect of primary infestation on subsequent resistance against different type of attackers will increase our insight in what is happening under ecologically relevant conditions. Some studies have already adopted such an approach (Shiojiri et al., 2002; Rodriguez-Saona et al., 2005; Moayeri et al., 2007; De Boer et al., 2008), and as observed in Chapter 7, previous infestation with a specific herbivore can affect subsequent herbivores in different ways. Besides aboveground herbivory, this has also been shown for other types of plant attackers. Belowground herbivory has been shown to influence aboveground insects at several trophic levels, through changes in secondary metabolites (Soler et al., 2005). Pathogen-induced phytochemical changes can affect both herbivore and parasitoid behavioural responses (Cardoza et al., 2003). The results in Chapter 6

with the *Trichoderma viride*-derived elicitor also indicate that fungal compounds can induce plant defence responses and subsequently affect interactions with other community members, like parasitoids.

Most studies have focused on vegetative plants, but because many plant species depend on pollination for reproduction, the effect of induced defence in flowering plants on pollination is an interesting subject. When plants start flowering, many plant traits change, such as morphology, resource allocation and volatile emission. As both flowering and induced defences require investment of carbon and nutrients, this could result in a trade-off between defence and pollination (Herms and Mattson, 1992). Several studies already indicated that root and leaf herbivory can influence pollinator behaviour (e.g. Lehtilä and Strauss, 1997; Hambäck, 2001; Poveda et al., 2003), however, the mechanisms underlying this phenomenon have not yet been elucidated (Kessler and Halitschke, 2007). The effects of changes in the flowering plant on interactions with herbivores and parasitoids as well as the changes due to herbivory in attraction of pollinators provide an interesting research field.

Jasmonate use to activate defence responses has been suggested as a method to improve pest control (Powell and Pickett, 2003; Ibrahim et al., 2005; Pickett et al., 2007). JA treatment of plants has been shown to reduce oviposition preference and development of herbivores and to result in parasitoid attraction (e.g. Chapters 3 and 4; Thaler, 1999a; Birkett et al., 2000; Thaler et al., 2002a), which both will benefit pest control. Herbivore-infested JA-treated plants are more attractive than JA-treated plants without herbivores, which implies that parasitoids can still effectively find their host in JA-treated plots (2004). Moreover, treatment with a low JA dose that by itself does not induce predator attraction can result in an increased attraction of predators to prey-infested plants (Gols et al., 2003). However, to benefit crops, the application of JA should not have negative side effects for mutualists. Chapter 5 shows that for black mustard plants pollinators are not negatively influenced by JA treatment. This suggests that JA could improve pest control. However, also wild relatives of crop species have been shown to have more effective direct and indirect defences (e.g. Loughrin et al., 1995; Bukovinszky et al., 2005). Volatiles from wild relatives have been shown to be emitted in larger amounts or to be more attractive to parasitoids. Possibly selection for higher defence levels in crops can also serve as an effective control measure, even in combination with JA.

In recent years an array of techniques and new insights into mechanisms of plant defence became available from which insect–plant interaction research can benefit. Following up on the use of an array of elicitors, recent advances have been made using inhibitors (Chapter 7; D'Alessandro et al., 2006; Mumm et al., 2008) to study the importance of terpenoids and shikimic acid-derived and LOX-dependent volatiles for parasitoid attraction. These studies illustrate the potential of inhibitors for use in behavioural studies. Furthermore, the knowledge and methodology

gained with model species such as *Arabidopsis thaliana*, can be exploited in brassicaceous crops and wild species, profiting from molecular and chemical studies and techniques available to measure gene expression, secondary metabolites, phytohormones and genetic modification (e.g. Broekgaarden et al., 2007; Zheng et al., 2007). Recent findings for other model species on the early events of plant defence induction, recognition of attackers and triggering of signalling pathways (Maffei et al., 2007a), should be taken further to the behavioural level. Building on the knowledge of molecular, chemical and bio-assay techniques so far used in laboratory and greenhouse experiments and applying this to studies of increasingly complex interactions, in field studies, with multiple infestation and flowering plants, will further promote the understanding of induced defence in a community ecology context (Snoeren et al., 2007; Chapter 2, Bruinsma and Dicke, 2008).

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SAMENVATTING

Plantenverdediging

Planten kunnen tijdens hun leven worden aangevallen door verschillende belagers, zoals insecten en ziekteverwekkers. Planten hebben verschillende strategieën ontwikkeld om zich te verdedigen tegen deze belagers. Deze verdedigingsmechanismen kunnen altijd aanwezig zijn in de plant, onafhankelijk van belagers, of induceerbaar zijn, dat wil zeggen alleen aanwezig als de plant aangevallen wordt. In dit proefschrift heb ik gekeken naar induceerbare chemische verdedigingsmechanismen van planten tegen plantenetende insecten en de reacties van verschillende insecten op deze chemische veranderingen in de plant. Verdedigingsmechanismen die effect hebben op de planteneters noemen we directe verdediging en als de verandering natuurlijke vijanden van de planteneters aantrekt, noemen we dat indirecte verdediging. Het was al bekend dat vraat door planteneters chemische verdedigingsmechanismen van planten behorende tot de Brassicaceae (kruisbloemigen) induceert. Ik heb elicitoren en remmers van verschillende stappen van de processen die leiden tot geïnduceerde verdediging gebruikt, om te onderzoeken hoe veranderingen in geïnduceerde verdediging interacties tussen planten en insecten beïnvloeden.

Figuur 1. Spruitkoolplant (boven) en mosterdplant (onder) (foto's: Tibor Bukovinszky)

Studiesysteem

Het studiesysteem dat ik gebruikt heb om deze interacties te bestuderen, bestond uit planten die tot de kruisbloemigen behoren en uit insecten die op verschillende manieren gebruik maken van de plant. Ik heb de meeste experimenten die in dit proefschrift beschreven staan uitgevoerd met spruitkoolplanten (*Brassica oleracea* var *gemmifera*; Figuur 1a). Voor het onderzoek beschreven in hoofdstuk 5 had ik bloeiende planten nodig. Hiervoor heb ik mosterdplanten (*Brassica nigra*; Figuur 1b) gebruikt, omdat deze, in tegenstelling tot spruitkool, in het eerste jaar bloeien. Ik heb de interacties tussen deze planten en drie specialistische planteneters bestudeerd. Specialistische planteneters eten alleen van planten behorende tot een specifieke groep planten, in dit geval planten met glucosinolaten. Glucosinolaten zijn chemische afweerstoffen die door specialistische planteneters ook gebruikt kunnen worden voor waardplantherkenning. Deze stoffen komen voornamelijk voor in kruisbloemige planten. Ook heb ik een aantal natuurlijke vijanden, sluipwespen, van deze planteneters bestudeerd. De sluipwespen leggen hun eitjes in rupsen (Figuur 2) en de larven ontwikkelen zich vervolgens in de nog levende rups. De sluipwespen kunnen hun gastheer vinden door middel van geuren die de plant uitscheidt. Ze reageren op geuren afgegeven door planten met vraatschade door rupsen. Daarnaast heb ik de reacties van bestuivers van mosterdplanten geobserveerd.

Figuur 2. Een sluipwesp vrouwtje legt haar eitjes in een rups (foto: Tibor Bukovinszky)

Fenotypische manipulatie

Als de verdediging van de plant wordt geïnduceerd, verandert de chemie en daarmee het fenotype (de verschijningsvorm) van de plant. De inductie van verdedigingsmechanismen kan worden gemanipuleerd door toepassing van elicitoren en remmers. Dat noemen we fenotypische manipulatie. Elicitoren en remmers zijn chemische stoffen die de verdedigingsreactie van de plant beïnvloeden. Hierdoor kan een verdedigingsreactie geïnduceerd of juist geremd worden. Door een specifieke stap van de reactie te manipuleren, kan de rol hiervan in de interacties tussen plant en insect worden onderzocht.

Figuur 3. Het kleine koolwitje legt enkele eitjes op een koolblad (boven) (foto: Tibor Bukovinszky) terwijl het grote koolwitje een heel eipakket legt (onder) (foto: Hans Smid).

Jasmonzuur

Jasmonzuur is een belangrijk plantenhormoon in de zogenaamde octadecanoid reactieketen. Deze keten is betrokken in plantenverdediging tegen vraat door planteneters. Het aanbrengen van dit plantenhormoon op een plant kan inductie van een verdedigingsmechanisme tot gevolg hebben. Deze reactie van de plant lijkt op (maar is niet hetzelfde als) de chemische verandering als reactie op beschadiging door planteneters. In hoofdstuk 3 staat beschreven hoe de behandeling van spruitkoolplanten het gedrag van planteneters beïnvloedde. Twee specialistische planteneters, de vlinders *Pieris rapae* en *P. brassicae* (respectievelijk het kleine en het grote koolwitje; Figuur 3) legden hun eitjes liever op planten die niet geïnduceerd waren door jasmonzuurbehandeling dan op planten die wel geïnduceerd waren. Ook de ontwikkeling van *P. rapae* van 1^{e} stadium rups tot pop duurde langer op geïnduceerde planten dan op ongeïnduceerde planten. Dit suggereert dat het vermijden van het leggen van eitjes op geïnduceerde planten een adaptieve eigenschap is, die gunstig is voor de ontwikkeling van de nakomelingen van de vlinders. De hoeveelheid glucosinolaten in extracten van het bladoppervlak boden geen verklaring voor de geobserveerde eilegvoorkeur.

Door jasmonzuur geïnduceerde veranderingen in de plant beïnvloedden niet alleen planteneters, maar ook de vijanden van de planteneters (hoofdstuk 4). Het was al bekend dat sluipwespen geuren van door planteneters belaagde planten kunnen herkennen en deze gebruiken om hun gastheren (rupsen) te vinden om eitjes in te leggen. Ook de behandeling met jasmonzuur trok sluipwespen aan tot de plant. In dit proefschrift wordt beschreven dat deze aantrekking van de sluipwespen afhankelijk is van de jasmonzuurdosis en de tijd tussen behandeling en het moment van aanbieden van de planten aan de sluipwesp. Jasmonzuurbehandeling had echter niet hetzelfde effect als rupsenvraat. De geurstofemissie van planten met vraatschade was anders dan die van planten die behandeld waren met jasmonzuur. Hoewel planten die behandeld waren met jasmonzuur grotere hoeveelheden geurstoffen uitscheidden, hadden de sluipwespen een voorkeur voor de planten met vraatschade ten opzichte van de met jasmonzuur behandelde planten.

Alamethicine

Ionkanaalactiviteit en veranderingen in de ionbalans spelen een rol in een vroeg stadium van de verdedigingsreactie. Alamethicine is een elicitor die geïsoleerd is uit de schimmel *Trichoderma viride*. Het is een ionkanaalvormend peptidemengsel en in hoofdstuk 6 van dit proefschrift gebruikt om plantenverdediging te induceren. Behandeling van planten met alamethicine resulteerde in een voorkeur van sluipwespen voor deze planten boven ongeïnduceerde planten. Hoewel behandeling met alamethicine resulteerde in veel lagere geurstofemissie dan jasmonzuurbehandeling, maakten de sluipwespen geen onderscheid tussen beide behandelingen. Kwaliteit van de geurstofemissie lijkt dus belangrijker voor het zoekgedrag van de sluipwespen dan kwantiteit.

Remmers

Naast chemische inductie van verdedigingsmechanismen kan ook chemische remming gebruikt worden voor onderzoek naar de impact van de verschillende stappen in inductie van plantenverdediging tegen vraatschade. Het gebruik van remmers biedt de mogelijkheid specifieke stappen in de inductie van verdediging te remmen, terwijl de visuele kenmerken van een beschadigde plant, zoals de vraatschade, wel aanwezig zijn. In hoofdstuk 7 is phenidone gebruikt om de chemische verdedigingsreactie van de plant te remmen. Phenidone remt het enzym lipoxygenase dat een sleutelrol vervult in de eerdergenoemde octadecanoid reactieketen.

Behandeling met phenidone vlak voor de start van rupsenvraat verminderde de aantrekking van sluipwespen tot de planten. Ook planteneters reageerden op de beïnvloeding van de octadecanoid reactieketen. Twee planteneters die verschillen in eilegvoorkeur, reageerden ook op phenidonebehandeling maar op verschillende wijze. *Pieris brassicae*, het grote koolwitje, legde haar eitjes bij voorkeur op onbeschadigde bladeren. Maar na behandeling met phenidone maakte ze geen onderscheid meer tussen beschadigde en onbeschadigde bladeren. *Plutella xylostella*, de koolmot (Figuur 4), legt haar eitjes juist bij voorkeur op beschadigde bladeren en behandeling met phenidone verminderde deze voorkeur. Deze resultaten geven aan dat de eilegvoorkeur van deze specialistische planteneters een chemische basis heeft en het remmen van de octadecanoid reactieketen hun gedrag beïnvloedt.

Figuur 4. Koolmot (foto: Tibor Bukovinszky)

Bloeiende planten

Het meeste onderzoek aan induceerbare plantenverdediging is gedaan aan niet-bloeiende planten. Aangezien zowel verdediging als reproductie beide kostbare processen zijn, waarvoor energie en nutriënten nodig zijn, kan een conflict ontstaan tussen de investeringen in beide processen.

Figuur 5. Zweefvlieg (boven) (foto: Tibor Bukovinszky) en honingbij (onder) (Hans Smid).

Vraatschade door planteneters aan bladeren, bloemen en wortels kan bestuiversbezoek beïnvloeden, maar de mechanismen hierachter zijn nog onbekend. In hoofdstuk 5 is het effect van jasmonzuurbehandeling op nectarsecretie en bestuiversbezoek aan bloemen onderzocht. Bloemen van planten die behandeld waren met jasmonzuur scheidden minder nectar uit dan bloemen van controle-planten en planten met rupsenvraat, maar de suikerconcentraties van de nectar veranderden niet. Ook het aantal en de duur van de bezoeken door zweefvliegen en honingbijen (Figuur 5) veranderden niet door jasmonzuurbehandeling.

Conclusie

De resultaten van het onderzoek beschreven in dit proefschrift geven de complexiteit van geïnduceerde plantenverdediging weer en de verscheidenheid van gedragsveranderingen van insecten van zowel verschillende trofische niveaus als binnen een trofisch niveau. Een aanpak zoals beschreven in dit proefschrift, gebaseerd op fenotypische veranderingen veroorzaakt door gebruikmaking van elicitoren en remmers, in combinatie met moleculair-genetische technieken en toepassing van recente ontwikkelingen in fytochemie, biedt een interessante benadering om tot beter begrip te komen van de complexe interacties tussen planten en insecten en van de rol die informatiestoffen hierbij spelen.

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DANKWOORD

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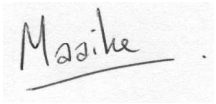

CURRICULUM VITAE

Maaike Bruinsma was born on 17 April 1978 in Leiderdorp in The Netherlands. After finishing secondary school in Leiden in 1996, she started studying biology at Leiden University, where she specialised in ecology. During her MSc study, she studied the role of sexual selection and thermal effects on mating success of different colour morphs of two-spotted ladybirds. In a second project, she investigated the response of two generalist herbivores to the diversity of pyrrolizidine alkaloids in a range of *Senecio* species; part of the study was carried out at the University of Chile in Santiago, Chile. After completion of her MSc in Biology in 2001, she left Leiden and started working at the Netherlands Institute of Ecology in Heteren, The Netherlands. There, she first worked on an extensive literature study and organised a three-day international workshop, both on the effects of genetically-modified plants on soil ecosystems, and later contributed to the start-up phase of a large project on nature restoration on ex-arable land. After these two projects, she worked at an environmental advice agency, 'De Straat milieuadviseurs' in Delft on a project aimed at transferring soil quality information from paper archives into an easily-accessible database for several city councils in The Netherlands. After a short period of working on a project on the control of potato late blight in organic farming systems at the Crop and Weed Ecology group of Wageningen University, she started her PhD at the Laboratory of Entomology in Wageningen in January 2004. The results of this research, carried out under the supervision of Marcel Dicke and Joop van Loon, on phenotypic manipulation of plant defences and its effects on several members of the associated insect community, are described in this thesis. After defending her PhD dissertation, she will continue working on insect–plant interactions within the Laboratory of Entomology, in a post-doc project focussing on possible trade-offs for plants between induced defence and pollination.

PUBLICATIONS

Peer-reviewed publications

- Bruinsma, M., IJdema, H., Van Loon, J.J.A., and Dicke, M. (in press) Differential effects of jasmonic acid treatment of *Brassica nigra* on the attraction of pollinators, parasitoids and butterflies. *Entomologia Experimentalis et Applicata*.

- Bruinsma, M. and Dicke, M. (2008) Herbivore-induced indirect defence: from induction mechanisms to community ecology. In: A. Schaller (ed.) *Induced plant resistance to herbivory*. Springer Verlag, Berlin, Germany, pp. 31-60.

- Zheng, S.J., Van Dijk, J., Bruinsma, M., and Dicke, M. (2007) Sensitivity and speed of induced defense of cabbage (*Brassica oleracea* L.): dynamics of *BoLOX* expression patterns during insect and pathogen attack. *Molecular Plant-Microbe Interactions* 20: 1332-1345.

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Other publications

- Dicke, M., Bruinsma, M., Bukovinszky, T., Gols, R., De Jong, P.W., Van Loon, J.J.A., Snoeren, T.A.L., and Zheng, S.J. (2006) Investigating the ecology of inducible indirect defence by manipulating plant phenotype and genotype. *IOBC/wprs Bulletin* 29 (8): 15-23.

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(To be) Submitted

- Bruinsma, M., Posthumus, M.A., Mumm, R., Van Loon, J.J.A., Dicke, M. Jasmonic acid-induced changes in *Brassica oleracea* attract parasitoids: effects of time and dose and differences with induction by herbivores.

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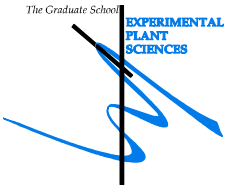
- Bruinsma, M., Poelman, E.H., Van Broekhoven, S., Posthumus, M.A., Mueller, M.J., Van Loon, J.J.A., and Dicke, M. Effect of the lipoxygenase-inhibitor phenidone on plant response to herbivore feeding and behavioural responses of parasitoids and specialist herbivores.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Maaike Bruinsma
Date: 09 May 2008
Group: Laboratory of Entomology, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Phenotypic manipulation of odour production and effects on interactions with community members	15 Jun 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Herbivore-induced indirect defence: from induction mechanisms to community ecology	2006
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days 2004, Vrije Universiteit Amsterdam	2004
EPS PhD student days 2005, Radboud University Nijmegen	2005
EPS PhD student days 2007, Wageningen University	2007
► **EPS theme symposia**	
EPS Theme 2 Symposium 2005, Leiden University	2005
EPS Theme 2 Symposium 2007, University of Amsterdam	2007
► **NWO Lunteren days and other National Platforms**	
Entomologendag, Ede	2005 & 2006
Netherlands Ecological Research Network 1st Annual Meeting, Lunteren	2008
► **Seminars (series), workshops and symposia**	
Entomology Seminar Series	2004-2008
Current Themes in Ecology, Wageningen	2006
EPS Ecology Symposium, Wageningen	2007
► **Seminar plus**	
► **International symposia and congresses**	
IOBC workshop on induced resistance, Delemont, Switzerland	2004
International Society of Chemical Ecology 21st Annual Meeting, Washington, USA	2005
Measuring behaviour, Wageningen	2005
4th biannual IMPRS PhD Symposium, Jena, Germany	2006
Symposium on Insect-Plant Interactions 13, Uppsala, Sweden	2007
► **Presentations**	
Poster, IOBC workshop on induced resistance, Delemont, Switzerland	2004
Oral presentation, ISCE 21st Annual Meeting, Washington, USA	2005
Oral presentation, 4th biannual IMPRS PhD Symposium, Jena, Germany	2006
Oral presentation, EPS Theme 2 Symposium, Amsterdam	2007
Oral presentation, SIP 13, Uppsala, Sweden	2007
► **IAB interview**	2006
► **Excursions**, PhD excursion UK	2007
Subtotal Scientific Exposure	*16.1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Spring School Chemical Communication	2005
Advanced Statistics	2006
Plant-Insect Interactions Workshop	2006 & 2007
► **Journal club**	
Bi-weekly PhD lunch meetings and monthly insect-plant interactions discussion group	2004-2008
► **Individual research training**	
Subtotal In-Depth Studies	*6.6 credits**

4) Personal development	*date*
► **Skill training courses**	
Presentations Skills	2005
PhD Competence Assessment	2005
Grant Proposal Writing	2007
NWO Talent days, Utrecht, Den Haag	2007
► **Organisation of PhD students day, course or conference**	
Organisation of PhD excursion to United Kingdom	2007
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36.4**

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 credits

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

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