Temporal dynamics of induced responses in *Brassica juncea*

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Chapter 1

General Introduction

Picture by: Sonia Dourlot

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Plant resistance to insects

The diverse distribution of insect herbivores among their host plants and its effect on the development and defence strategies of plants has long been a subject of interest for ecologists. To establish themselves on a plant, insects possess several strategies to overcome the plant's defence barriers, allowing them to feed, grow and reproduce on it. In response, plants have developed a stunning array of morphological and chemical defences to deter, kill or delay attacking organisms before they are able to cause extensive damage (Schoonhoven *et al.* 1998). Various studies have been conducted to show that morphological barriers such as hairs, spines, cuticle, trichomes, lignified vascular bundles, and accumulation of abrasive minerals such as silica may be insuperable for herbivorous insects and thus physically prevent them from feeding on plant tissue (Peeters 2002, Agerbirk *et al.* 2003, Clauss *et al.* 2006, Hanley *et al.* 2007, Keathley and Potter 2011). Indeed, plant structural traits have been shown to affect insect feeding, performance and diversity (Peeters *et al.* 2007, Randlkofer *et al.* 2010).

Additionally, plants produce a number of chemical compounds to resist insect attack (Bernays 1977, Schoonhoven *et al*. 1998, Simmonds 2001). Most of these compounds are not part of primary processes such as growth, reproduction, photosynthesis or protein production, and are therefore termed as secondary metabolites. Nevertheless, they play a major role in various biotic interactions, for example in the attraction of pollinators and as defences against herbivores. More than 100,000 plant secondary compounds, such as phenolics, terpenoids, alkaloids, cyanogenic glucosides and glucosinolates have been identified (Hadacek 2002, Howe and Schaller 2008). However, each plant species produces only a small, but unique combination of these compounds. Some of these compounds are always expressed; even when the plant is not under attack by herbivores (Wittstock and Gershenzon 2002). These chemical defences are called constitutive defences. Ehrlich and Raven (1964) emphasized their significance as the product of coevolution between plants and herbivores. In

environments with high herbivore densities, plants producing secondary chemicals that make their tissues unpalatable or toxic to herbivores have an advantage over other plants (Fraenkel 1959, Dethier *et al.* 1960). However, compounds that inhibit feeding by some herbivores (generalists) may be used by others (specialists) as feeding stimulants (Agrawal and Karban 1999). Thus, the diversity of both morphological defences and of secondary compounds is one line of evidence suggesting that herbivores significantly influence the evolution of plants (Jones and Firn 1991).

Constitutive defence mechanisms are 'static' and account for an overall resistance against generalist herbivores. However, once attacked by insects or pathogens, plants can also apply an 'active' defence strategy by altering the concentrations of existing secondary compounds or induce the *de novo* production of defensive structures and chemicals (Harborne 1988). Such responses may be either restricted to the location where the damage occurred (Agrell *et al.* 2003, de Vos *et al.* 2006), but are often triggered systemically throughout the plant, thus affecting the defence levels of unattacked plant parts as well, both aboveground (Mattiacci *et al.* 2001, Zangerl 2003) and belowground (Bezemer *et al.* 2004, van Dam *et al.* 2004, Kaplan *et al.* 2008). This systemic induction can be attributed to signalling molecule(s) that are transported between different plant tissues (Baldwin *et al.* 2001). Induced responses may be termed as 'induced resistance' if they alter the behaviour of the herbivores by affecting their performance and/or preference (Karban and Myers 1989). These resistance mechanisms not only present physical and chemical barriers that directly affect their attackers, but also include strategies of indirect defences by employing predators and parasitoids (Dicke and Vet 1999, Rasmann *et al.* 2005, Agrawal 2007, van Dam 2009a, van Dam *et al.* 2010, Heil 2011b). Moreover, induction due to aboveground damage may affect the herbivores feeding belowground and *vice versa* (Van der Putten *et al.* 2001, van Dam and Raaijmakers 2006, Soler *et al.* 2007a, van Dam *et al.* 2009).

While constitutive defences increase plant fitness when the herbivores are abundant in the environment, the energy invested in such defences can reduce plant fitness when herbivores are absent (Agrawal *et al.* 1999). For example, constitutive defences such as tannins and glandular trichomes can be costly because plants have to heavily invest their resources in building these structural traits at the expense of growth and reproduction (Sagers and Coley 1995, Hare *et al.* 2003). Inducible defences have evolved to reduce these costs by allowing the plant to invest in defence only when necessary, but avoid costly allocations to defence when herbivores are absent (Agrawal and Karban 1999). However, these changes may provide a selective advantage only if the plant fitness due to induced defences exceeds its fitness with only constitutive defences under different environmental conditions (Strauss *et al.* 1999, Cipollini *et al.* 2003, van Dam and Heil 2011). Whether these phenotypic responses may increase or decrease a plant's fitness largely depends on the reliability of environmental cues that predict environmental changes, as well as on the phenotypic and genetic costs associated with the plant's responses (Karban *et al.* 1999, van Dam and Baldwin 2001).

Induced responses: one stimulus, many responses

Herbivore feeding may induce a range of morphological and/or physiological responses in a plant. These responses are observed both at the phenotypic as well as at the molecular level (Karban and Baldwin 1997, Agrawal 1999) (Figure 1.1). Multiple defences may guard the plant from a wider range of attacking organisms as compared with individual defensive mechanisms. Chemical defences, for instance, are often effective against generalist herbivores but can be circumvented by specialists (Poelman *et al.* 2008a, Poelman *et al.* 2011), whereas mechanical defences may protect plants against specialists as well (Traw and Dawson 2002b). Thus, functional multiplicity of induced traits, comprising of physiological requirements of the plant and a wide spectrum resistance against various herbivores and pathogens, may enhance the functionality of these resistance mechanisms (Koricheva *et al.* 2004).

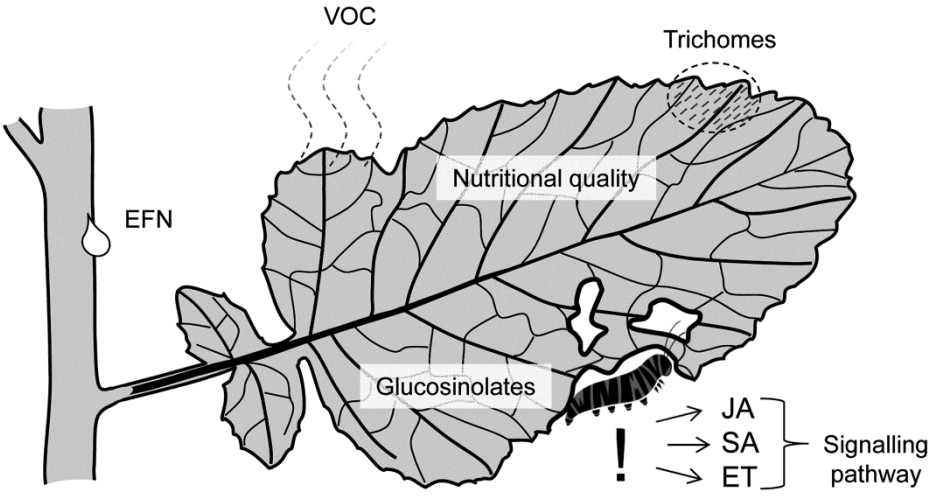

Figure 1.1: Schematic drawing of a *Brassica juncea* leaf in which it is shown how induced responses to herbivory are expressed at both phenotypic and molecular levels. The induced responses can confer direct resistance, through the induction of glucosinolates, trichomes and changes in nutritional quality; or indirect resistance, via the induction of volatile organic compounds (VOC) and extrafloral nectar (EFN). The phenotypic responses are determined by cross-talk between signalling pathways the jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) pathways (Picture by Onno Calf).

Direct resistance mechanisms

Direct resistance mechanisms mainly rely on morphological features, secondary metabolites or primary metabolites of the plant. Following insect damage, the induction of hairs, spines and trichomes has been observed (Myers and Bazely 1991, Agrawal 1999, Traw and Dawson 2002a, Hanley *et al*. 2007). Moreover, cell wall thickening after insect attack can also decrease the digestibility of plant tissues to insects with chewing mouthparts (Clissold *et al.* 2004). By fortifying these structural traits, plants enhance mechanical barriers to insect probing and feeding (Traw and Dawson 2002b).

In addition, plants also increase the production of constitutively present secondary compounds or produce new compounds following herbivory (Karban and Baldwin 1997, Chen 2008). These compounds principally belong to three

classes: (1) phenolics (such as tannins, flavonoids and lignin), (2) terpenoids (such as limonene and eucalyptol), and (3) nitrogen containing compounds (such as glucosinolates, cyanogenic glycosides and benzoxazinoids). When induced, these compounds directly affect the palatability or nutritive value of the plant. (Jahangir *et al.* 2009), or they may act as feeding deterrents or toxins (Baldwin 1988a, Hopkins *et al.* 2009, Jansen *et al.* 2009).

Resource allocation to primary functions and defence allocation are not independent. In fact, the resources for the biosynthesis of secondary compounds are derived from the conversion of primary metabolites (Balandrin *et al.* 1985). Besides, plants may also deal with herbivory by tolerating damage through altering their primary metabolism across the whole plant (Boege 2004). Schwachtje and Baldwin (2008) suggest that this strategy may have four reasons: (i) investing in secondary metabolites, that are heightened after herbivore attack, may be costly and the resources are relocated from production of primary metabolites towards these induced chemicals (Smith and Stitt 2007, Bolton 2009), (ii) allocating primary metabolites away from the organ that is under attack may safeguard resources in herbivore-inaccessible tissues for future plant regrowth (Utsumi and Ohgushi 2007, Steinbrenner *et al.* 2011), (iii) primary metabolites, e.g. trehalose, may serve as a signal in the defence signalling pathway (Ahn and Lee 2003, Ahn *et al.* 2007, Bolton 2009) and (iv) primary metabolites may themselves have a defensive function (Lou and Baldwin 2004, Howe and Schaller 2008). Another advantage of altering primary metabolites in response to herbivory would be that if the feeding herbivore is confined to individual host plants, its growth may be reduced by consuming low quality foliage on induced plants (Thaler *et al.* 1999, van Dam *et al.* 2000), and thus it would be more susceptible to predators and parasitoids.

Indirect resistance mechanisms

Besides resisting the damaging insects, plants may also indirectly defend themselves by enhancing the effectiveness of the natural enemies of herbivores.

This mutualistic "enemy of my enemy" strategy is aimed at reducing enemy pressure by attracting, nourishing and/or housing herbivore parasitoids, predators and protective ants (Heil 2008). In general, two main indirect resistance mechanisms are employed:

1. Extrafloral nectar (EFN) production: Plants bearing EFN are widely distributed around the world. Around 114 plant families with more than 700 genera and 4000 species of plants are known to possess EFN (Keeler 2008). EFN plays an important role in the plant's constitutive and induced indirect defences against root or shoot herbivores by providing an alternative food source for parasitoids, predators and mutualistic ants (Wackers and Bezemer 2003, Heil 2004, Heil *et al.* 2004, Lach *et al.* 2009, Heil 2011a, Holland *et al.* 2011, Escalante-Pérez and Heil 2012). Although most of the studies have been done in the context of ant–plant interactions (Bentley 1976, Heil *et al.* 2001, Bixenmann *et al.* 2011), several studies also addressed the role of EFN in attracting parasitoids (Stapel *et al.* 1997, Lewis *et al.* 1998, Rose *et al.* 2006). The presence of freely available carbohydrates and amino acids, along with other nutrients, makes EFN a wholesome nutritional resource for the herbivore's enemies, thus enhancing their retention on the plant. The fitness advantage that plants obtain when they attract herbivore's natural enemies is demonstrated in many studies (Kessler and Baldwin 2004, Rosumek *et al.* 2009, Romero and Koricheva 2011). Hence, the availability and accessibility of food sources, such as nectar in a target area, can strongly enhance host-finding efficiency and reproductive success of the natural enemies of herbivores (Stapel, *et al.* 1997, Arimura *et al.* 2005, Rose, *et al.* 2006).

   Other structural traits involved in indirect defences are cellular food bodies, which provide ants with an exclusive food source, and domatia, which provide housing for predators. Their development or induction is not known to respond to herbivory. However, food body production may increase in the presence of mutualists (Heil 2008).

2. Volatile organic compound emission: Plants emit an array of volatile organic compounds (VOCs). VOCs are emitted from different parts of the plant such as leaves, flowers, fruits and roots. Constitutively they provide a reproductive advantage by attracting pollinators and seed dispersers. Many of these compounds are released in larger quantities when the plant is damaged, serving as direct defence against pathogens and herbivores by deterring them, and indirectly by attracting their natural enemies (Dicke and Vet 1999, Baldwin 2010). The quality and quantity of the volatile blends are influenced by the interactions with biotic factors (Vet 1999), such as herbivore-derived elicitors (Arimura *et al.* 2005) and microorganisms (Cardoza *et al.* 2002, Leitner *et al.* 2008). In several cases, it is shown that the herbivores' oral secretions contain elicitors that trigger the signalling pathways inducing volatiles synthesis genes, thus causing VOC blends that are different from intact or mechanically damaged plants (Mattiacci *et al.* 1995, Alborn *et al.* 1997, Pare and Tumlinson 1997, Dicke and van Loon 2000). VOCs may be induced by herbivores feeding aboveground (Dicke 1999, Maes and Debergh 2003, Erb *et al.* 2010) or belowground (Bezemer *et al.* 2004, Soler *et al.* 2007b, van Dam *et al.* 2010, Soler *et al.* 2012b) as well as by oviposition (Hilker and Meiners 2002, Hilker and Meiners 2011). The plant hormone jasmonic acid and its precursors are known to act as signals in plants induced by herbivore feeding and some studies show that the induction of VOCs can be mimicked by the application of jasmonic acid (Boland *et al.* 1995, Karban and Baldwin 1997). However, the volatile blends induced by these compounds may not be identical to those induced by herbivore damage (Bruinsma *et al.* 2009, Hare 2011). In addition to biotic factors, VOC production rate is also affected by abiotic factors such as temperature and light (Gouinguené and Turlings 2002, Maes and Debergh 2003), carbon dioxide and ozone concentration (Vuorinen *et al.* 2004, Himanen *et al.* 2009) and UV radiation (Eichholz *et al.* 2011).

Once induced, VOCs may directly affect the feeding herbivores by acting as repellents and thus altering their behaviour (De Moraes *et al.* 2001, Halitschke *et al.* 2008, Snoeren *et al.* 2010), or indirectly by attracting the natural enemies of the feeding herbivores (Turlings *et al.* 1990, Vet and Dicke 1992). VOCs are also known to mediate communication within and between plants, as well as affect other arthropods and microorganisms (Dicke and Vet 1999, Heil and Ton 2008, Baldwin 2010). Therefore it has been proposed that the ecological significance of herbivore-induced VOCs is probably more complex than initially thought (Takabayashi and Dicke 1996). One of the well-documented indirect defence strategies of plants against herbivores is the herbivore-induced emission of specific VOC blends that attract carnivorous natural enemies (Sabelis and Vandebaan 1983, Vet and Dicke 1992, Dicke and Vet 1999, Paré and Tumlinson 1999, Arimura *et al.* 2000, Dicke and van Loon 2000, Pierre *et al.* 2011). It has been shown in plants of several families that these compounds permit predators or parasitoids to discriminate damaged plants from their intact counterparts (Turlings *et al.* 1990, Dicke *et al.* 1999, Shiojiri *et al.* 2001, Soler *et al.* 2007, van Dam *et al.* 2010). Herbivores themselves generally release few VOCs because they are under strong selection to be inconspicuous (Vet *et al.* 1991). Plant VOCs may be more detectable for the natural enemies and can also be reliable cues because species-specific compounds or specific ratios of ubiquitous compounds are induced by specific herbivore attack (Vet and Dicke 1992). Hence, plant produced herbivore-induced volatiles provide detectable and reliable signals to foraging predators and parasitoids (Vet *et al.* 1995, Vet 1999).

Induced responses and hormonal signalling

Recent breakthroughs in molecular biology have provided scientists with exciting new tools to pursue eco-genomics research in the field of induced plant resistance. Numerous studies have shown that herbivory causes variation in phytohormones concentrations, which activates a series of signalling events,

resulting in altered gene expression profiles (Arimura *et al.* 2000, Hermsmeier *et al.* 2001, Bodenhausen and Reymond 2007, Dicke and Baldwin 2010, Verhage *et al.* 2011). Many signalling pathways contribute to these responses. The pathways leading from stimulus to end response are part of complex signalling networks, with overlaps and interlinks, commonly referred to as "cross-talk" (Schenk *et al.* 2000, Traw and Bergelson 2003, De Vos *et al.* 2005, Beckers and Spoel 2006). In this process, different signalling pathways act synergistically or antagonistically and regulate the plant's response to the damage.

The two major signalling pathways involved in mediating a wide range of biotic stress responses are the jasmonic acid (JA) and the salicylic acid (SA) pathway. Infestation by chewing insects or by necrotrophic pathogens usually triggers the JA pathway, while sucking insects or biotrophic pathogens generally activate the SA pathway (Conrath *et al.* 2006, Kost and Heil 2006, Arimura *et al.* 2009, Koo and Howe 2009, Ament *et al.* 2010, Verhage *et al.* 2011, Soler *et al.* 2012a). Upon insect attack, JA is rapidly synthesized via the oxylipin biosynthesis pathway (Wasternack 2007, Gfeller *et al.* 2010). The responses mediated by JA and its derivatives seem to be regulated different branches of the JA signalling pathway. These branches are specific according to the nature of herbivory and show limited overlap, suggesting that the context in which the JA signal is perceived is crucial in fine-tuning the JA response (De Vos *et al.* 2005, Lorenzo and Solano 2005, Kazan and Manners 2012).

However, the story is more complex than this simple distinction between the JA and SA pathways. Some arthropods and pathogens induce both the signalling pathways, and cross-talk between JA and SA pathways is also commonly observed (Bostock *et al.* 2001, Cipollini *et al.* 2004). Moreover, in the last decade, cross-talk between the signalling pathways of plant hormones JA, SA, ethylene (ET) and abscisic acid (ABA) emerged as an additional regulatory mechanism of plant immunity (Clarke *et al.* 2000, Bostock 2005, Adie *et al.* 2007, Robert-Seilaniantz *et al.* 2011). SA, ET and ABA may act as important differential regulators of the JA response (Pieterse *et al.* 2009, Leon-Reyes *et al.* 2010). SA

generally acts antagonistically on the JA pathway, while ET and ABA regulate different branches of the JA response (Verhage *et al.* 2010, Verhage *et al.* 2011). The blend of hormonal alarm signals varies greatly in quantity, composition and timing (Koornneef and Pieterse 2008). These 'signal signatures' create a specificity of the plant induced responses that allows the plant to fine-tune its defence response to the attacking herbivore species (Reymond and Farmer 1998, Reymond *et al.* 2000, De Vos *et al.* 2005).

Responses to more than one attacker

On the transcriptional, biochemical, and organismal levels, induced plant responses can be specifically associated with the feeding insect species (Travers-Martin and Müller 2007, Erb *et al.* 2011, Pierre *et al.* 2011). However, in nature, plants are commonly attacked by more than one herbivore species at the same time (van Dam 2009b, Bukovinszky *et al.* 2012). Understanding the manner in which plants cope with these simultaneously occurring attacks and the resulting interactions within the context of their ecological community can be critical for understanding the insect-plant interactions (Shiojiri *et al.* 2001, Dicke *et al.* 2009, Rodriguez-Saona *et al.* 2010, Soler *et al.* 2012a).

It has been demonstrated that damage by more than one herbivore induces responses in a plant that differ from responses induced by a single herbivore (de Boer *et al.* 2008, Pierre *et al.* 2011, Soler *et al.* 2012a). Plants respond to multiple attacks in three possible ways: (1) the response is additive due to a lack of specificity of responses to different herbivores; (2) the response is synergistic, whereby the plant responds to each herbivore differently but the response is strengthened when the plant is damaged by two species simultaneously, and (3) the response is antagonistic, where the plant response is attenuated when the plant is dual-damaged (Rodriguez-Saona *et al*. 2010). Induced plant responses to more than one attacker may cause direct (e.g. through facilitation or competition) or indirect (e.g. via plants or carnivores) interactions between the attacking herbivores (Kessler *et al.* 2004, Kaplan and Denno 2007, Dicke *et al*. 2009).

Therefore, unravelling the effects of herbivory, as well as interactions between induced responses on resistance traits is critical in order to understand the ecological mechanisms controlling the distribution of insect herbivores.

Timing and coordination of plant responses

Induced plant responses usually peak only for a certain period of time. A single herbivore induces many different responses, and each response may have its own temporal dynamics. It is expected that the timing and localisation of each response is strongly correlated with its primary function. For example, responses that are meant for wound repairing and immediate protection of the plant against further damage may be rapid (Maffei *et al.* 2007) (Figure 1.2a). On the other hand, compounds that are produced *de novo* may take more time to be expressed as they require activation of a biosynthetic pathway which involves the activation of genes and protein synthesis and transporting the signal before the defence compound can be expressed (Baldwin *et al.* 1994b, Kant *et al.* 2004). Hence, for these responses, there may be a certain time lag before they are significantly increased over base levels (Figure 1.2b). The lag phase may be for a short duration varying from hours (Schultz 1988, Maffei *et al*. 2007) to days (Hopkins *et al*. 2009) or may even extend for months or years (Trapp and Croteau 2001, Boege 2004, Poelman *et al*. 2008a). The strength of the induced responses comes down gradually to basal or 'static' levels and analogous to the activation of response, response decay may also show temporal variation of hours to days after the herbivore damage (Baldwin *et al.* 1994a, Anderson *et al.* 2001, Agrell 2003, Chung *et al.* 2008). Therefore, inducible response kinetics often show a bell shaped curve with the initial build-up or lag phase, a peak period and a gradual decrease to basal levels (Schultz 1988) (Figure 1.2c). The duration of activation, peak and decay of responses in relation to the dynamics of herbivore feeding determines the effectiveness of induced defences (Gomez *et al.* 2010). This

general phenomenon cannot be applied to structural responses because they cannot easily be catabolised and return to basic levels after induction on existing leaves (Figure 1.2d). However, newly formed leaves exhibit reduced production of these structures once the herbivore attack ceases (Björkman *et al.* 2008).

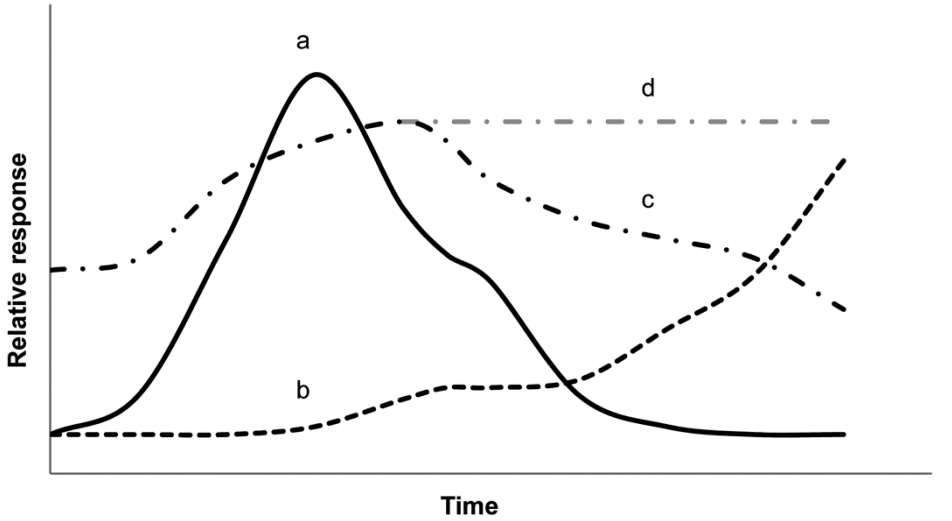

Figure 1.2: Schematic representation of temporal dynamics of different induced responses to herbivory. (a) depicts the dynamics of rapidly responding compounds that returns to basal level in a short time (e.g. volatiles); (b) represents responses that take time to attain peak levels but are long lasting (e.g. structural responses). (c) and (d) depict secondary structures or compounds that are already constitutively present, but increase in concentration following herbivory. The chemical compounds (c; e.g. glucosinolates) gradually decrease back to basal level, but structural responses (d; e.g.: Trichomes) cannot be easily catabolized to basal levels. (Picture by Onno Calf)

Due to such temporal variations in responses, studies that followed the time course of induced responses have found that the implications of plant's induced resistance may be suffered by the damaging organism itself, either directly (De Moraes *et al.* 2001, van Dam *et al.* 2001), or indirectly via the attraction of natural enemies (De Moraes *et al.* 1998, Arimura, *et al*. 2005). Besides, a herbivore species that is spatially or temporally separated from the damaging species may bear the cost because of the response delay (Agrawal 2000b, Agrawal

2001, Bezemer and van Dam 2005, Pierre *et al*. 2011). Thus, determining the time interval between various changes occurring in the plant as well as their coordination and function in time is essential to understand the role of each induced response in direct or indirect defences.

Consequences of induced responses: "one man's meat is another man's poison"

The effect of induced plant responses on herbivores have been demonstrated across a broad range of taxa (Karban and Baldwin 1997). Direct and indirect induced responses can alter both behaviour and development of the attacking herbivores. Physiological changes following herbivory can reduce the nutritional quality of plant tissue (Baldwin 1988b, Bukovinszky *et al.* 2009) and/or increase levels of defence compounds and structures at the same time (van Dam and Raaijmakers 2006, Travers-Martin and Müller 2007). Together, these induced responses may have an adverse effect on insect feeding, growth and development (Karban and Baldwin 1997, Mattiacci *et al*. 2001, Agrell *et al.* 2003, van Dam *et al.* 2005, Soler *et al*. 2007, Hopkins *et al.* 2009). Additionally, the plant may be avoided by some insects searching for high quality host plants (De Moraes *et al*. 2001, Kessler and Baldwin 2001). However, increased levels of defence may lead to both resistance as well as susceptibility of the plant, depending on the herbivore species. For example, phytochemicals that are implicated in resistance in generalists may serve as attractants and oviposition stimulants by specialists (Poelman *et al*. 2008a, Hopkins *et al*. 2009). In some cases, the defences may be sequestered by specialist herbivores for their own defence (Müller *et al.* 2001, Hartl and Baldwin 2006, Muller and Sieling 2006). Molecular studies support the suggestion that insect herbivores and pathogens have evolved to manipulate plants to their own benefit by modulating the plant's defence signalling network (Voelckel *et al.* 2001, Cipollini, *et al*. 2003, Pieterse and Dicke 2007). For instance, components in the oral secretion of the generalist herbivore *S. exigua* suppressed JA- dependent defences in

Arabidopsis thaliana through the activation of the SA pathway (Weech *et al.* 2008, Diezel *et al.* 2009). Similarly, elicitors from salivary excretions of *Helicoverpa zea* reduced the JA-dependent synthesis of nicotine produced by tobacco plants (Musser *et al.* 2005). Thus, induced defence responses may have contrasting effects on insects with similar and different feeding strategies (Agrawal 2000b, Wittstock *et al.* 2003, Van Zandt and Agrawal 2004, Soler *et al.* 2009, Poelman *et al.* 2010). Whether these responses increase or decrease a plant's fitness depends on the spectrum of herbivores in the community as well as on the phenotypic and genetic costs associated with the responses (Karban *et al.* 1999, van Dam and Baldwin 2001, Strauss *et al.* 2002). Therefore, behavioural studies measuring orientation, feeding, oviposition and performance of the herbivores are important to determine the effect of induced responses on the insect in question.

Ecological and economic significance of studies on induced responses

Herbivorous insects are economically important, especially in agricultural systems, where they may reduce the net productivity by 50% and under the worst of circumstances, may completely wipe out the agricultural crop. Losses due to insect herbivores are estimated to be around 10-20% for major crops globally (Pimentel 1991, Ferry 2004). Chemical insecticides that were used earlier in order to manage these insects were found to cause serious health hazards to humans and other animals. Moreover, they cause problems such as environmental pollution, pesticide resistance and secondary pest outbreak. To significantly reduce the use of pesticides, ecological pest management makes use of an array of complementary methods such as physical, cultural and biological control practices (van Lenteren and Woets 1988). Additionally, various alternative strategies to protect crops from insects by exploiting endogenous resistance mechanisms are turning out to be crucial to the success of these agricultural endeavours.

Recent research suggests that understanding the mechanisms by which plants cope with environmental stresses such as herbivory in their natural environment may provide new ways to enhance resistance to pests in an ecologically and economically sound manner. In fact, a large number of behavioural, chemical and evolutionary ecologists, plant physiologists and crop scientists are making research efforts to understand the mechanisms, selective pressures and ecological consequences of these interactions (Turlings *et al*. 1990, von Dahl *et al.* 2006, Wurst *et al.* 2008, Zhang *et al.* 2008, van Dam *et al.* 2010). Moreover, there is an increased interest in comprehending the implications of induced plant signalling in the light of agricultural pest control (Dicke and Baldwin 2010, Soler *et al*. 2012a). Thus, gaining more knowledge on induced plant responses is important for plant ecology as well as for pest control and maintenance of productivity in agriculture. Information in this area will be important for both forms of research, *i.e*., basic research concerning physiology, ecology and evolution of defence mechanisms of plants, as well as applied research devoted to the development of agricultural tools for crop protection.

Aim and hypotheses

The aim of my study is to investigate the temporal changes in the direct and indirect induced traits that occur in Indian mustard (*Brassica juncea*) leaves due to herbivory. I tested the following hypotheses:

- The induction of plant responses varies in time.
- The induction of structural plant responses is slower than that of chemical plant responses.
- Induced plant responses to single and double herbivory are dissimilar.
- Generalists and specialist herbivores induce specific responses in plants, which are perceived differently by the later arriving species.
- Induced plants are resistant to generalists but susceptible to specialist herbivores, but resistance and susceptibility are dependent on the dynamics of the induced response.

- Induced volatile blends change temporally and these changes affect the attraction of specialist and generalist herbivores and their parasitoids differentially in time.
- The temporal dynamics of the induced responses are reflected in the dynamics of the concomitant gene expression profiles.

"The (mustard) bomb squad": Brassicaceae as a model system

Annual species belonging to the family Brassicaceae (formerly Cruciferae) are particularly suitable for addressing the differential induction of resistance for several reasons. These plants are easy to germinate and grow, and their resistance traits have been well recognized (Vaughn and Boydston 1997, Mithen 2001, Traw and Dawson 2002b, Felkl *et al.* 2005, Bennett *et al.* 2006, Hopkins *et al*. 2009, Jahangir *et al*. 2009, Müller *et al.* 2010). Plants of this family have also been extensively studied and much is known about their interaction with generalist and specialist herbivores (Kostal 1992, Agrawal 2000a, Traw and Dawson 2002b, van Dam *et al.* 2003, Hopkins *et al*. 2009, Müller *et al*. 2010). Moreover, *Arabidopsis*, which is a model plant for molecular, physiological and genetic studies, belongs to this family. Since the sequencing of this species is already successfully completed, molecular studies of related plant species is facilitated.

Glucosinolates: The "Mustard oil bomb"

Plants belonging to the family Brassicaceae contain several classes of defensive secondary compounds such as proteinase inhibitors (Cipollini and Bergelson 2000, Cipollini 2002), saponins (Shinoda *et al.* 2002) and anthocyanins (Rostás *et al.* 2002). However, the most studied defence secondary compounds in this family are the glucosinolates (Cole 1976, Mithen 2001, Wittstock and Halkier 2002, Field *et al.* 2004, Hopkins *et al*. 2009, van Dam *et al*. 2009).

Members of the Brassicaceae are well characterized by the presence of the glucosinolate defence mechanism, the so-called 'mustard oil bomb' (Matile

1980). These compounds are one of the most extensively studied groups of secondary metabolites with more than 120 different structures described (Fahey *et al.* 2001, Yan and Chen 2007). This sulfur- and nitrogen- containing group of compounds shares a common core structure, a sulfonated oxime and aβ - thioglucose moiety, which is linked to variable side chains originating from different amino acids (Grubb and Abel 2006, Halkier and Gershenzon 2006). Much of the diversity amongst glucosinolates arises from the addition of different sized alkyl groups to the side chain of the amino acids, principally valine, phenylalanine and methionine. Based on the chemical structure of their side chain, glucosinolates may be subdivided in different classes, such as aliphatic, aromatic and indole glucosinolates (Fahey, *et al*. 2001).

Glucosinolates occur in all parts of the plant (Wittstock and Halkier 2002). Although intact glucosinolates themselves may deter generalist herbivores (Li *et al.* 2000), the product they form upon plant damage are thought to be most effective. When plant cells rupture, they come in contact with myrosinase, an enzyme stored in specialized cells (Rask *et al.* 2000). This results in the formation of different types of even more potent hydrolysis products. According to the side chain and the reaction conditions, combination of myrosinase with the glucosinolates results in several different noxious and toxic products, such as isothiocyanates, thiocyanates, oxazolidine- 2- thiones, epithionitriles and nitriles (Wittstock and Gershenzon 2002). These products can mediate insect behaviour and can have deterrent or toxic effects on generalists (Clauss *et al*. 2006, Hopkins *et al*. 2009). In contrast, several specialists are attracted to the volatile isothiocyanates and stimulated by the non-volatile glucosinolates for feeding and oviposition (Renwick 2002, Poelman *et al.* 2008b). Also, some specialists have developed ways to overcome these compounds. For example, Muller and Sieling (2006) found that the specialist *Athalia rosae* sequesters glucosinolates and hence its performance remains unaffected by high glucosinolate or myrosinase levels of *Brassica juncea*. Another study found that larvae of the specialist *Plutella xylostella* inhibit production of toxic glucosinolate-hydrolysis products by converting

glucosinolates into desulfoglucosinolates by means of a sulfatase. The desulfoglucosinolates cannot be degraded by myrosinases and are excreted with the faeces (Ratzka *et al.* 2002). Thus, glucosinolates may have different effects depending on the insect species (Stadler and Reifenrath 2009).

Glucosinolates are present constitutively in plants, but the production of the 'mustard oil bomb' parts is induced by herbivore feeding. Plant injury affects all types of glucosinolates, but their specific effect is dependent on the species of the plant, its age, the extent of damage, time delay between damage and observation as well the tissue studied (Birch *et al.* 1992, Bodnaryk 1992, Birch *et al.* 1996, van Dam and Raaijmakers 2006, Martin and Muller 2007, van Dam and Oomen 2008, Hopkins *et al*. 2009). Following herbivore damage or simulated damage, the amounts of certain glucosinolates are typically induced by several-fold (Textor and Gershenzon 2009). Glucosinolate induction by herbivory has been documented for a number of plant species (Birch *et al.* 1990, van Dam and Raaijmakers 2006, Martin and Muller 2007). The three major types of glucosinolates, i.e., aliphatic, aromatic and indolic, often respond differently to herbivory because they are formed from different amino acid precursors. In addition, the enzymes and other regulatory factors involved in their biosynthesis may be dependent on the gene activation, resulting in their differential production (Halkier and Gershenzon 2006, Sonderby *et al.* 2010).

"Spiny spices": Significance of trichomes in Brassicaceae

Trichomes are considered as 'soft' weapons for insect defence as compared to other lethal defences (Dalin *et al.* 2008). They are hair- like structural elements of the epidermis of the plant that hinder the insects by impeding their locomotion, feeding and oviposition (Levin 1973). Trichomes are known to provide physical protection against herbivores in mustard species (Agren and Schemske 1993, Palaniswamy and Bodnaryk 1994, Agrawal 1999, Agrawal 2004, Clauss *et al*. 2006). Consequently, experimental removal of trichomes increases damage by both specialist and generalist herbivores (Lamb 1980).

While most plants produce trichomes constitutively, some species respond to damage by increasing trichome density or the number of trichomes increase following damage (Agrawal 1999, Traw and Dawson 2002a, Traw and Bergelson 2003). An increase in trichome density could reflect either an increase in trichomes per leaf or a decrease in leaf area of the new leaf (Karban and Baldwin 1997, Traw and Dawson 2002a). Apart from reducing overall damage, induced trichome production may divert the insects from feeding on the most valuable parts of the plant, such as the newly formed younger leaves. Such variation in trichome density between different parts of the plant may reflect developmental constraints, but may also be advantageous since damage to older leaves is likely to be less detrimental to the plant than damage to the young apical leaves (Feeny 1976). Trichome production can only be induced in leaves that are still developing during or subsequent to attack because plants cannot change the density of trichomes on fully grown leaves (Traw and Bergelson 2003). The induction of trichomes may be expressed in new leaves within days or weeks after the initial attack (Agrawal 1999, Dalin *et al*. 2008). Thus, observing structural traits such as trichomes may provide a better insight into the systemic resistance mechanisms of Brassicaceae.

Role of volatiles

In the plants of the mustard family, volatiles that make-up the characteristic *Brassica* 'odour' consist of glucosinolate hydrolysis products, such as (iso)thiocyanates, along with compounds belonging to various groups, such as alcohols, ketones, aldehydes, esters, nitriles, terpenoids, sulfides and carboxylic acids (Tollsten and Bergstrom 1988, Geervliet *et al.* 1997). These volatiles may serve both to deter unwanted herbivores (Landolt 1993) and to attract beneficial insects such as pollinators and parasitoids (Soler *et al*. 2007a, Bruinsma *et al.* 2008). Moreover, specialists feeding on Brassicaceae have also developed strategies to utilize these stimuli as a cue for finding their host plants (Agrawal and Sherriffs 2001, Bruinsma *et al.* 2010).

Herbivore damage results in the emission of volatile blend of about 20 to over 200 compounds (Vaughn and Boydston 1997, Smid *et al.* 2002, Dicke *et al.* 2003, Dicke and Baldwin 2010, Pierre *et al*. 2011). There is ample evidence that carnivores selectively exploit damage-induced plant volatiles in Brassicaceae for locating their herbivorous hosts or prey in greenhouse and field conditions (Geervliet *et al.* 1994, Geervliet *et al.* 1998, Dicke and Vet 1999, Bukovinszky *et al.* 2005, Poelman *et al.* 2009, Snoeren *et al*. 2010, van Dam *et al*. 2010). For example, in a laboratory study, the specialist parasitoid *Cotesia plutellae* preferred odours of damaged cabbage plants to the odours of intact cabbage in Y-tube olfactometer tests (Vuorinen *et al*. 2004). In another study, aphid-induced volatiles released from *Arabidopsis thaliana*, *Brassica juncea*, *B. rapa* and two cultivars of *B. napus* attracted the predator *Coccinella septempunctata* (Girling and Hassall 2008). However, volatile changes due to herbivory by the cabbage root fly *Delia radicum* feeding on *B. nigra* deterred *C. glomerata*, a parasitoid of the leaf herbivore *Pieris brassicae* (Soler, *et al*. 2007b). When parasitoids *C. glomerata*, *C. rubecula*, and *Diadegma semiclausum*, differing in host range and host specificity, were tested for their attraction to volatiles from herbivore-induced, JA-induced, and non-induced plants in a time and dose-dependent study, all three species preferred herbivore-induced plants over JA- induced and control plants (Bruinsma *et al*. 2009).

These studies emphasized the importance of quantity as well as quality of the volatiles in the host-location behaviour of the wasps. Variation in herbivore-induced volatile composition after herbivory may be relevant to the parasitoids, since it provides evidence on the herbivore identity and its suitability to serve as a prey (Vet and Dicke 1992, Vet 1999). In plant-parasitoid interaction studies, it is therefore essential to identify and quantify the chemicals mediating those interactions, to elucidate the factors affecting their release, and to obtain information on the time course of their emission.

The study system

Plant species

Brown mustard or Indian mustard (*Brassica juncea* L. Czernov) is an annual herb of the family Brassicaceae. It is said to be a native of Asia and is also widely distributed in Canada, Argentina, Australia, Fiji, Mexico, and the United States. It is cultivated particularly in India and neighbouring countries, as well as in southern Russia, Central Africa, and north of the Caspian Sea for its variable, glabrous, rather thin basal leaves which are eaten raw as salad or cooked. Although widely and extensively grown as a vegetable, it is more often grown for its seeds which yield an essential oil and are used to produce condiments. This oil is one of the major consumable oils in India. However in much of Europe, black mustard (*B. nigra*) has replaced Indian mustard as the original source of commercial mustard seed. In addition, it is known for its medicinal importance and is considered as a folk remedy for arthritis, foot ache, lumbago, and rheumatism (Duke and Wain 1981).

Brown mustard acts as a host for many insects such as the Diamondback moth (*Plutella xylostella*), mustard sawfly (*Athalia proxima*), tobacco cutworm (*Spodoptera litura*), beet armyworm (*S. exigua*) and various aphids and leaf miners. Prominent resistance strategies for protection against these insects and various pathogens in *B. juncea* include glucosinolates, trichomes and volatiles that may be repellent to many pests and may serve as attractants to predators and parasitoids.

Insect species

Pests

The tobacco cutworm, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), is distributed worldwide. This species is a generalist and feeds on wide variety of plants belonging to 40 different families including Malvaceae, Solanaceae, Poaceae and Brassicaceae (Salama *et al.* 1970, Brown and Dewhurst 1975). Its main host plants include castor, cotton, tomato, mint, cabbage and mustard. Due to the polyphagous nature of *S. litura* and development of resistance to several

insecticides, this insect is used as a model for various behavioural, physiological, biochemical, molecular and genetic studies.

The beet armyworm, *Spodoptera exigua* (Hüb.), also belongs to the family Noctuidae. Although it has a Southeast Asian origin, it now has a cosmopolitan distribution. *S. exigua* has a wide host range, and is a serious pest of crops such as alfalfa, beet, broccoli, cabbage, cotton, mustard, peanut, potato and tomato (Capinera 2001). It is a popular model as a generalist herbivore pest in many studies on induced responses (Bezemer *et al.* 2003, Voelckel and Baldwin 2004, Maischak *et al.* 2007, Van Zandt 2007).

The Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a destructive insect pest of Brassicaceous crops globally. It causes considerable crop losses throughout the world and alone requires on an average US$ 1.0 billion in annual management costs (Talekar and Shelton 1993). Due to extensive use of insecticides and severe selection pressure, it has developed high levels of resistance to many broad spectrum insecticides, and is now considered to be among insect pests that have aroused interest and concern globally (Mota-Sanchez *et al.* 2002, Sarfraz and Keddie 2005). Hence, an ecological approach with reduced input of insecticides can serve as an important tool for *P. xylostella* management.

The large cabbage butterfly, *Pieris brassicae* (L.) (Lepidoptera: Pieridae), is a specialist leaf chewing butterfly that feeds on many species of wild and cultivated and is commonly found throughout Europe, Africa and Asia to the Himalayas. It was earlier thought to sequester glucosinolates from the *Brassica* plants, but recent studies did not find the evidence of sequestration in these butterflies (Müller *et al.* 2003). Nevertheless, larvae of *P. brassicae* are known to feed exclusively on plants containing glucosinolates and particularly sinigrin acts as a feeding stimulant to this butterfly (Renwick *et al.* 1992).

The green peach aphid, *Myzus persicae* (Sulz.) (Hemiptera: Aphididae), is a cosmopolitan generalist feeding on hundreds of host plants in over 40 plant

families such as Solanaceae, Compositae, Cucurbitaceae and Brassicaceae (Hill 1983). These aphids feed by sucking sap from their hosts, and a prolonged infestation can cause appreciable reduction in yield of root crops and foliage crops, resulting in high economic losses. Besides, aphids are known to vector many plant viruses, which is potentially the greatest consequence of aphid infestations. In fact, *M. persicae* is considered by many to be the most important vector of plant viruses throughout the world with over 100 viruses transmitted by this species. Nymphs and adults are equally capable of virus transmission (Kennedy *et al.* 1962, Namba and Sylvester 1981). The aphid vectors diseases in more than 30 plant families, including beans, sugar beet, sugarcane, *Citrus*, tobacco and *Brassica* spp. (Hill 1983).

The cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae), as the name suggests, is a specialist root chewing herbivore that feeds on several species of the family Brassicaceae. Females lay eggs in the soil around the stem of the plant. Newly hatched larvae feed by boring galleries in the upper roots. As much as 300 larvae may be found on one plant, due to which the inner parts of the main root are severely damaged. This causes disruption in water and nutrients transport to the stem and leaves, thus resulting in the death of the plant. Besides weakening of the root, larval feeding also causes secondary infections by root- rot pathogens (Soroka *et al.* 2004).

Parasitoids

The genus *Cotesia* belongs to the order Hymenoptera and family Braconidae. Many species from this genus act as biological agents for pest control and are used in studies related to tritrophic interactions and parasitoid population genetics and physiology.

C. marginiventris (Cress.) is a general larval parasitoid of Noctuid pests. It is known to parasitize many serious generalist pests such as black cutworm (*Agrotis ipsilon*), corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*), soybean looper (*Pseudoplusia includens*), cabbage looper (*Trichoplusia ni*),

southern armyworm (*Spodoptera eridania*), fall armyworm (*S. frugiperda*), tobacco cutworm (*S. litura*) and beet armyworm (*S. exigua*) (Sourakov and Mitchell 2001). Due to its generalist nature, it is a model parasitoid and a potentially important biological control agent (Tillman 2001).

C. plutellae (Kurd.) is a major solitary, larval endoparasitoid of the Diamondback moth, *P. xylostella*. It has been established as a biocontrol agent for the pest and is recommended for Diamondback moth management (Talekar and Shelton 1993). It is known to be host-specific and is attracted to plants induced only by *P. xylostella* (Shiojiri *et al.* 2000).

C. glomerata (Linn.) is a gregarious endoparasitoid of a few genera of the family Pieridae, with *P. brassicae* and *P. rapae* as their preferred hosts. Females lay about 20- 30 eggs per larval host. *C. glomerata* is resistant to attack by many hyperparasitoids and is an important vector in the transmission of the granulosis virus in cabbageworm (Levin *et al.* 1983).

Diaeretiella rapae (McIntosh) (Hymenoptera: Braconidae) is a cosmopolitan parasitoid of many species of aphids, including those infesting Brassicaceae crops. It is a solitary endoparasitoid of adult and immature stages of aphids and is known to be primarily attracted to cruciferous plant odours (Vaughn *et al.* 1996).

Trybliographa rapae (Westwood) (Hymenoptera: Figitidae) is the main parasitoid of *D. Radicum* (Fournet *et al.* 2004). It is a solitary larval-pupal endoparasitoid. The parasitoid larva and the host larva have a parallel development until the host dies within its puparium and an adult parasitoid emerges (Harvey 2005).While foraging, *T. rapae* are attracted to volatiles emitted from infested plants (Brown and Anderson 1999). Although the host larvae are feeding belowground, the olfactory cues have been suggested to originate from both below- and aboveground parts of the plant (Neveu *et al.* 2002).

Outline of the thesis

In my thesis, I present an analysis of the temporal dynamics of various direct and indirect responses in Indian mustard, *Brassica juncea*, due to herbivory by the

generalist *Spodoptera* spp., the specialist *P. xylostella* or both of these herbivores together. Morphological (leaf size and trichome densities) and chemical (volatiles, glucosinolates, amino acids, sugars) responses were analysed over a temporal range of 24 hours to 20 days. The effect of direct systemic responses was assessed using a specialist (*P. xylostella*) and a generalist (*S. litura*) herbivore. In addition, to investigate indirect responses, orientation experiments were conducted on the generalist (*C. marginiventris*) and specialist (*C. plutellae*) parasitoids. Results were validated with expression analysis of genes that are involved in signalling and biosynthetic pathways governing these responses.

For the first time, the presence of extrafloral nectar (EFN) in Brassicaceae is being reported. Their sugar and amino acid contents were measured in five different varieties of *B. juncea* and the indirect function of these EFN droplets was assessed by testing their inducibility and attraction and survival of parasitoids on them.

These studies show that although morphological responses are slower than chemical responses, they also contribute to induced plant resistance in a relatively short time span. Moreover, before considering induced responses as resistance factors, their effect should be assessed at different points in time with both generalist and specialist herbivores. Therefore, extensive qualitative and quantitative analyses of temporal and spatial variations and system-wide assessment of induced responses in plants will accelerate the identification of specific targets that are critical for plant defences. High throughput validation and transgenic techniques will also bring improved crop plants with enhanced resistance using the identified targets for agriculture.

Chapter 2: Temporal dynamics of induced responses in *Brassica juncea* and their effect on generalist and specialist herbivores

In this chapter, I determined the temporal dynamics of various systemically induced responses occurring in *Brassica juncea* leaves after insect herbivory in India and the Netherlands (NL). Both morphological (trichomes, leaf size) and chemical (glucosinolates, amino acids, sugars) responses were analysed at

various time points between 4 and 13 days after damage by the generalist *Spodoptera spp*. The effect of systemic responses was assessed by studying the behaviour of a specialist and a generalist lepidopteron herbivore. I tested the hypotheses that morphological responses were slower than chemical responses, and that generalist herbivores would be more affected by induced responses than specialists.

Chapter 3: Dealing with double trouble: consequences of single and double herbivory in *Brassica juncea*

This chapter focuses on how single and double herbivory affects induced responses, and whether the temporal patterns of these responses have divergent effects on successive herbivores. Morphological (leaf length, area and trichomes), and chemical changes (leaf alkenyl and indole glucosinolates) in *Brassica juncea* were evaluated four, ten, fourteen and twenty days after damage by the specialist *Plutella xylostella* alone, or together with the generalist *Spodoptera litura*. Preference and performance of both herbivores were tested on these time points to understand an overall association between the damaging insects, various plant responses due to this damage and the herbivore insects facing the consequences over a period of time.

Chapter 4: Temporal dynamics of induced volatiles in *Brassica juncea*: chemical, molecular and ecological aspects

In Chapter 4, I applied behavioural, chemical and molecular tools to investigate if the temporal dynamics of herbivore- induced volatiles influence the preference of generalist and specialist herbivores and their parasitoids. Since volatiles are supposed to be one of the fastest induced responses, orientation preference of two different herbivores and their parasitoids was examined for *Brassica juncea* plants that had been induced by *Spodoptera spp.* for 24, 48 and 72 hours. Thereafter, I measured volatile production and also analysed gene expression to understand the underlying mechanism for the observed temporal preferences.

Chapter 5: Composition, structure and function of extrafloral nectaries in *Brassica juncea* (Brassicaceae)

In this chapter, I describe for the first time the presence of extrafloral nectaries in *Brassica juncea* and investigated their structure and possible function. Extrafloral nectaries have never been reported to occur in the family Brassicaceae to which *B. juncea* belongs. Morphology and period of occurrence of extrafloral nectar (EFN) in *B. juncea* plants were determined and histochemical studies were performed to observe the anatomy of the extrafloral nectaries. Additionally, sugar, amino acid and glucosinolate concentrations and composition of EFN was determined in five common varieties of *B. juncea*. The ecological function of these nectaries was investigated by studying the induction of EFN production after damage by main *Brassica* herbivores of different feeding guilds, *viz*. leaf chewing caterpillars, aphids and root herbivores. Parasitoids of each herbivore species were tested for attraction and survival on the nectar.

Chapter 6: General discussion

Here, I discuss how temporal dynamics of induced response may prove to be critical in determining the plant resistance strategies in its environment. I also discuss the future direction of research that could be useful in designing novel strategies for plant resistance in agriculture.

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Chapter 2

Temporal dynamics of herbivore-induced responses in *Brassica juncea* and their effect on generalist and specialist herbivores

Picture by: Vartika Mathur

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Abstract

Herbivore feeding may induce an array of responses in plants, and each response may have its own temporal dynamics. Precise timing of these plant responses is vital for them to have optimal effect on the herbivores feeding on the plant. This study measured the temporal dynamics of various systemically-induced responses occurring in *Brassica juncea* (L.) Czern. (Brassicaceae) leaves after insect herbivory in India and the Netherlands. Morphological (trichomes, leaf size) as well as chemical (glucosinolates, amino acids, sugars) responses were analysed. The effects of systemic responses were assessed using a specialist [*Plutella xylostella* L. (Lepidoptera: Plutellidae)] and a generalist [*Spodoptera litura* Fabricius (Lepidoptera: Noctuidae)] herbivore. We tested the hypotheses that morphological responses were slower than chemical responses, and that generalist herbivores would be more affected by induced responses than specialists. Glucosinolates and trichomes were found to increase systemically as quickly as 4 and 7 days after herbivore damage, respectively. Amino acids, sugars, and leaf size remained unaffected during this period. The generalist *S. litura* showed a significant feeding preference for undamaged leaves, whereas the specialist herbivore *P. xylostella* preferred leaves that were damaged 9 days before. Performance bioassays on generalist *S. litura* revealed that larvae gained half the weight on leaves from damaged plants as compared to larvae feeding on leaves from undamaged plants. These studies show that although morphological responses are somewhat slower than chemical responses, they also contribute to induced plant resistance in a relatively short time span. We argue that before considering induced responses as resistance factors, their effect should be assessed at different points in time with both generalist and specialist herbivores.

Introduction

Plants possess an array of morphological and chemical adaptations to reduce insect herbivory. Some of these defences are constitutively expressed. However, once an attack by insects or pathogens has occurred, plants may increase the concentrations of existing compounds and/or produce new structures and chemicals (Karban & Baldwin, 1997). The expression of these induced responses may be rapid for the immediate protection of the plant, or delayed because of activation of biosynthetic pathways. Studies that have examined induced responses on a temporal scale have mainly focussed on a single response (Wäckers *et al.* 2001; Rostás & Eggert, 2008; Dicke *et al.* 2009), or relatively short time scales of minutes, hours, or a few days after insect attack (Maffei *et al.* 2007; Hopkins *et al.* 2009). Consequently, little is known about the timing and expression of multiple defences over a longer time period of several days after attack.

Brown or Indian mustard, *Brassica juncea* (L.) Czern., belongs to the Brassicaceae family. Members of this family are characterized by the presence of glucosinolates. It is well documented that these secondary compounds increase following herbivory (Bodnaryk, 1992; Agrawal *et al.* 2002; van Dam & Raaijmakers, 2006), which is evident within days after the herbivore attack begins (Hopkins *et al.* 2009). Earlier studies have shown that artificial wounding increased the levels of alkenyl glucosinolates in cotyledons of 1-week-old *B. juncea* seedlings, whereas jasmonate application did not (Bodnaryk, 1992, 1994). Indole glucosinolates were not induced in young *B. juncea* plants that were subjected to wounding or hormone treatment (Bodnaryk, 1994). Herbivory by the glucosinolate-sequestering specialist herbivore, *Athalia rosae* (L.) on 5-week-old *B. juncea* plants, on the other hand, decreased glucosinolate concentrations locally after 1 day of feeding, especially in lines with high glucosinolates levels (Müller & Sieling, 2006). The contrasting results of these studies may be caused by the fact that glucosinolate responses were analysed in plants of different developmental stages that had been induced with different treatments and herbivores, and which were also analysed at different time spans after induction.

Besides producing chemicals, plants may also respond to herbivory by changes in morphological characteristics such as trichomes, thorns, spines, and hairs. *Brassica juncea* possesses trichomes as a constitutive morphological defence strategy. These are known to affect the feeding behaviour and performance of insects at various stages of their lives (Fernandes, 1994; Traw & Dawson, 2002b; Agrawal & Fishbein, 2006). Earlier studies have indicated that insect damage can increase the density or number of trichomes on new leaves of brassicaceous plants 2-3 weeks after herbivore damage (Agrawal, 1999; Traw, 2002; Traw & Dawson, 2002a). As glucosinolate levels may be induced within days following herbivore damage, these results suggest that the induction of trichomes is much slower than that of glucosinolates (Hopkins *et al.* 2009). Moreover, the various defence mechanisms do not act in isolation. In fact, the induction of multiple traits may provide a greater level of defence to the plant than would be possible if the traits were present independently (Steppuhn & Baldwin, 2008). Travers-Martin & Müller (2008) showed this in several species of the family Brassicaceae. Induced responses also include changes in primary metabolites, such as sugars and amino acids (van Dam & Oomen, 2008). Decreased nutritional value can also reduce herbivore performance, thereby contributing to plant resistance (Berenbaum, 1995).

The fact that every inducible defence has its own dynamics may have ecological consequences. Not only the organism that causes the damage may suffer the consequences (De Moraes *et al.* 2001; van Dam *et al.* 2001), but also later-arriving species may bear the cost because of a delay in the plant's response (Agrawal & Sherrifs, 2001; Poelman *et al.* 2008). Several studies have provided evidence for the adverse effect of induced plant defences on insect feeding, growth, and development (Karban & Baldwin, 1997; Mattiacci *et al.* 2001; Agrell *et al.* 2003; Soler *et al.* 2007). Furthermore, these defences may have antixenotic effects and result in avoidance by herbivorous insects (De Moraes *et al.* 2001;

Kessler & Baldwin, 2001). However, inducible compounds that inhibit feeding by generalists may be used as feeding and oviposition stimulants by specialists (Baoyu *et al.* 2001; Müller *et al.* 2001; Schoonhoven *et al.* 2005). Thus, induced defence responses may have contrasting effects on insects with different feeding strategies (Hopkins *et al.* 2009; Soler *et al.* 2009; Poelman *et al.* 2010). Knowledge of the temporal dynamics of chemical and morphological responses – together with changes in primary metabolism – is therefore important for understanding the eventual effects on various pest insects threatening *B. juncea* cultures in India.

In the present study, we combined all these aspects and determined the temporal changes in the morphological and chemical characteristics that occur after herbivore feeding in *B. juncea* leaves and examined their effect on generalist and specialist herbivores. The morphological responses that we measured consisted of trichomes and leaf size, whereas chemical responses included primary (sugars and amino acids) and secondary (glucosinolates) metabolites. These systemically-induced responses were analysed experimentally in plants that had been grown separately in two countries, the Netherlands and India, to determine the consistency of the induction pattern under different conditions. We used the generalist herbivores tobacco cutworm, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), in India and beet armyworm, *Spodoptera exigua* Hübner, in the Netherlands to induce the plants. Furthermore, the biological effects of the induced changes on the feeding preference and performance of the generalist *S. litura*, and preference of a specialist, diamondback moth [*Plutella xylostella* L. (Lepidoptera: Plutellidae)], were determined to establish whether these induced changes contribute directly to insect resistance towards the same and different herbivore species. We tested the following hypotheses: (i) chemical responses occur more quickly after herbivory than do morphological responses, and (ii) generalists will be more affected by induced chemical responses than specialists, whereas specialists are more affected by induced morphological characteristics.

Materials and methods

The experiments in the Netherlands were conducted in a glasshouse at the Netherlands Institute of Ecology (NIOO-KNAW), Heteren. In India, they were performed in an insect-free enclosure at Sri Venkateswara College, University of Delhi, Delhi.

Plants

Seeds of *B. juncea* var. *varuna* were obtained from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India, in 2008 and stored dry and in the dark at 10°C. Seeds were germinated on glass beads in water in 10×10 cm plastic containers with a clear plastic lid. In the Netherlands, the containers were placed in the greenhouse. The greenhouse was kept at 21°C during the day and 16°C at night, under ambient light conditions that were supplied with sodium lamps to maintain the minimum photosynthetically active radiation (PAR) at 225 μmoles $m^{-1}s^{-1}$ for at least 16 h. Seven days later, the seedlings were transferred to 2-l pots, containing 1200 g plain river sand. Plants were supplied with 0.5 Hoagland solution (Hoagland & Arnon, 1950) once a week. Every alternate day, five randomly chosen pots were weighed to determine the volume of solution needed to maintain the water content in the pots at 14% of the soil dry mass. The concentration of KH_2PO_4 in the 0.5 Hoagland solution was doubled in week 4 and tripled in weeks 5 and 6 to avoid nutrient deficiency.

In India, the plants were grown as mentioned above with the following differences. Due to lack of a temperature-controlled greenhouse, the plants were grown in an insect-free enclosure outside from October until beginning of March with a gap of 1 month from mid December to mid January. They were grown in garden soil in earthen pots and treated with 0.5 Hoagland solution weekly.

Insects

Egg batches of *S. exigua* were obtained from the Laboratory of Entomology, Wageningen University, the Netherlands. They were maintained in a climate

room at 27°C, 50-70% r.h., and L16:D8 photoperiod on an artificial diet (Vickerman & Trumble, 1999). In India, larvae of *S. litura* and *P. xylostella* were obtained from laboratory cultures at Sri Venkateswara College, New Delhi, maintained since 2008 on castor bean and cabbage leaves, respectively. The cultures were supplemented with individuals from the Department of Entomology, IARI (Pusa, New Delhi) to avoid inbreeding.

Induction of plants

All experiments were performed when the plants had 6-7 true leaves and were approximately 6 weeks old. For the experiments in the Netherlands, a single fourth instar *S. exigua* was introduced in a clip cage to the fourth leaf from the apex. The larvae were allowed to feed for 24 h. Control plants received empty clip cages. After 24 h, the larvae and clip cages were removed. In India, third instars of *S. litura* were used because in preliminary studies (V Mathur, unpubl.), it was found that the damage by third instar *S. litura* was approximately the same as that of fourth instar *S. exigua*.

Experimental and control plants were placed randomly on the tables. For measuring chemical responses, the third leaf of experimental and control plants was removed at 4, 6, 7, 8, 9, 10, 11, and 13 days after introduction of the larva. We started the period of our studies on day 4 because previous studies suggested that glucosinolate induction starts from the 3rd day after herbivore damage onwards (Hopkins *et al.* 2009). The leaves were immediately frozen and stored at -20°C until freeze-drying. Trichomes were counted and leaf size measured on days 4, 7, 9, 11, and 13 after damage on the abaxial as well as adaxial surface of the third leaves. Measurement of chemical and morphological responses were performed on separate sets of plants with equal (n=10) number of experimental and control plants for each day of the experiment.

Glucosinolates, amino acids, and sugars

All three chemical analyses were performed using the same leaf. Leaves were lyophilized and then ground with a ball mill. For the induction experiments

conducted in India, the ground leaf samples were sent to the Netherlands for extraction and high-performance liquid chromatography (HPLC) analysis. Finely ground plant material was dissolved in 1.0 ml 70% MeOH in water (vol/vol), vortexed, and immediately boiled for 5 min to kill remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 min and centrifuged at 58 g force. The extraction was repeated for the pellet except the boiling step. Both supernatants were combined per sample in a corresponding clean and labelled 2-ml Eppendorf tube to form a global methanol extract for glucosinolates, amino acids, and sugars. Each tube was supplemented individually with 70% MeOH to attain the average mass (assessed with three tubes) of a 2-ml Eppendorf tube containing 2.0 ml 70% MeOH. This 'stock' extract was stored at -20°C until further analysis.

Glucosinolate analysis was done according to van Dam *et al*. (2004). Half the stock extract (1.0 ml) was desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) and separated on HPLC by means of a reversed phase C-18 column with a CH_3CN-H2O gradient. Analysis was performed using a photodiode array (PDA) detector with 229 nm as the integration wavelength (Dionex, Sunnyvale, CA, USA). Desulfoglucosinolate peaks were identified based on their retention times and UV spectrum, based on glucosinolate standards (MPI Chemical Ecology, Jena, Germany; Community Bureau of Reference, Brussels, Belgium, code BCR-367R). To calculate glucosinolate concentrations in the plant tissue, the values obtained were multiplied by 2 before dividing by dry mass.

Sugar and amino acid analysis was performed according to van Dam & Oomen (2008). For soluble sugar analysis, 10 μl from stock solution was diluted with 990 μl MilliQ water and analysed on Dionex HPLC system, using a Carbopac PA1 column (2 ×250 mm) and a Carbopac PA1 guard column (2 ×50 mm) (Dionex, Sunnyvale, CA, USA). Separation of sugars was done with isocratic gradient mixture of 10% 1 M NaOH and 90% MilliQ water at a flow rate of 0.25 ml per min.

Amino acid analysis was done using 20 μl of the stock extract diluted in 980 μl MilliQ water. Amino acid concentration was analyzed on a Dionex HPLC system

by integrated pulsed amperometric detection. Amino acids were separated with a ternary gradient on a 2 ×250 mm AminoPac© PA10 column with a 2 ×50 mm AminoPac© PA10 Guard column (Dionex). The Sigma AA-S-18 amino acid standard (Sigma, St Louis, IL, USA) containing 18 amino acids was supplemented with asparagine, glutamine, and tryptophan (2.5 $\mu molesml^{-1}$ each) to obtain a reference sample containing the 20 most common amino acids.

Trichomes, leaf length, and leaf area

As trichomes were found only on the leaf veins, the number of trichomes on a 1-cm stretch was counted at four places on the adaxial as well as the abaxial surface using a dissecting microscope. Per leaf, the average number of trichomes per cm was calculated. These leaves were then scanned using a Hewlett Packard flatbed scanner (Hewlett Packard Company, Palo Alto, CA, USA). The scanned images were used to measure the length and area of the leaf using 'ImageJ' software with a global scale set to 59 pixels per cm (Rasband, 1997–2011; Abramoff *et al*., 2004).

Insect behaviour and performance bioassay

Based on the combined results of the induction experiments on the third leaf in the plants grown in India and the Netherlands, days 4 and 9 were selected to conduct insect behaviour and performance assays. These two time points after induction showed consistent increases for both glucosinolates and trichomes in the plants grown in both countries. All behavioural experiments were conducted in India. For each experiment, plants to be used on day 9 after induction were induced 5 days before the plants that were to be used at day 4 after induction, so that all the treatments of the experiment could be performed on the same day.

Feeding preference assays

A glass Petri dish of 20 cm diameter was lined with moist filter paper. Third leaves of each control plant and plants induced 4 and 9 days earlier were cut using scissors, outlined with a pencil on graph paper and placed at an equal distance

from each other in a Petri dish. Feeding preference assays were conducted with either two fourth instars of *P. xylostella* or one sixth instar or two third instars of *S. litura* in one Petri dish. Larvae were introduced in the centre of the Petri dish and allowed to move and feed freely on the three leaves. After 24 h, the larvae were removed and damaged portions of the leaves were outlined on graph paper. For each leaf, the total area consumed was calculated by comparing the missing leaf material with the outline of the undamaged leaf. Fifteen replicates were performed for *P. xylostella*, 22 for sixth instar, and 20 for third instar *S. litura* larvae.

Insect performance assays

Third leaves of control plants and plants induced 4 and 9 days earlier were removed, weighed, and placed individually in containers lined with moist filter paper (n = 15 per treatment). Five containers were kept without larvae to determine leaf moisture loss. Newly moulted penultimate instar *S. litura* larvae were starved for 4 h. After weighing the larvae to the nearest 0.001g, they were introduced individually to containers. The average larval body mass at the beginning of the experiment was comparable for all treatments (ANOVA: $P>0.8$). The larvae were allowed to feed on the leaf for 24 h, after which they were weighed to calculate the gain in body mass. The remainder of the leaf was removed and weighed as well. The remaining leaf mass at the end of each experiment was subtracted from the initial weight of the leaf. Change in leaf weight due to loss of moisture was determined from leaves in containers without larvae and deducted from the experimental and control leaves.

Waldbauer indices were calculated as follows: consumption index (CI) = [(leaf mass ingested)/(larval mass gain ×number of days)], approximate digestibility (AD) = [(leaf mass ingested – frass mass)/(leaf mass ingested)], efficiency of conversion of digested food (ECD) = [(larval mass gain)/(leaf mass ingested –frass mass)], and efficiency of conversion of ingested food (ECI)= [(larval mass gain)/(leaf mass ingested)] (Waldbauer, 1968).

Statistical analysis

Data were analysed using SPSS 16.0 (SPSS, Chicago, IL, USA). Glucosinolates, sugars, and amino acid data were √x-transformed to attain normality and homogeneity of variance. Normality and homogeneity of variance were determined using one-sample Kolmogorov-Smirnov test and Levene's test, respectively, on the residuals. The influence of 'treatment' and 'time after induction' on these metabolites was analysed using MANOVA. Exact *F*-values and corresponding probabilities for treatment and time after treatment were based on Pillai's trace. The relationship between damage and effect on these metabolites was investigated using univariate ANOVA. If differences were found to be significant, Bonferroni post-hoc tests were conducted. Differences in trichome densities on adaxial and abaxial surfaces were evaluated by similar methods. However, given that the data were normal and homogeneous, they were not transformed.

The data on larval feeding preference and performance could not be transformed to meet the assumptions of parametric analysis. Hence, the amount of leaf area consumed of each treatment was evaluated using a non-parametric χ^2 Friedman's test and the nutritional indices were analysed using non-parametric Kruskal-Wallis ANOVA.

Results

Glucosinolates, amino acids, and sugars

The major glucosinolates in *B. juncea* leaves were 3-butenyl glucosinolate or gluconapin (ca. 74%) and 2-propenyl glucosinolate or sinigrin (ca. 23%). Other minor glucosinolateswere 4-pentenylglucosinolate (glucobrassicanapin), 3-indolylmethyl glucosinolate (glucobrassicin), 4-methoxy-3-indolylmethyl glucosinolate (4-methoxyglucobrassicin), and 2-phenylethyl glucosinolate (gluconasturtiin). Of these, gluconapin, sinigrin, and glucobrassicanapin are alkenyl glucosinolates, and their values were combined, whereas glucobrassicin and 4-methoxy-glucobrassicin are indole glucosinolates (Table 2.1).

Table 2.1: Mean (± SE; n = 10) of total, alkenyl, gluconapin (GNA), sinigrin (SIN), and indole glucosinolates (lmoles g)1 dry mass) in experimental (E; damaged for 24 h by a *Spodoptera* spp. larva) and control (C) third leaves of *Brassica juncea* grown in The Netherlands and India. Days refer to number of days following insect damage

Days	Treatment	Total	Alkenyl	GNA	SIN	Indole
				The Netherlands		
4	E	82.87 ± 8.20	82.67 ± 8.20	64.50 ± 6.38	17.28 ± 2.06	0.11 ± 0.02
	C	50.70 ± 4.86	50.61 ± 4.85	39.20 ± 3.72	11.08 ± 1.28	0.07 ± 0.01
6	E	74.11 ± 4.58	73.93 ± 4.57	57.11 ± 3.23	16.11 ± 1.36	0.08 ± 0.01
	C	64.41 ± 3.51	64.30 ± 3.50	50.37 ± 2.60	13.17 ± 0.95	0.06 ± 0.01
7	E	71.34 ± 6.08	71.22 ± 6.07	54.16 ± 4.37	16.54 ± 1.84	0.06 ± 0.02
	C	50.00 ± 2.19	49.93 ± 2.18	39.90 ± 1.69	9.61 ± 0.62	0.03 ± 0.01
8	E	62.86 ± 4.99	62.75 ± 4.98	47.45 ± 3.32	14.52 ± 1.92	0.09 ± 0.01
	C	53.80 ± 5.80	53.70 ± 5.79	39.93 ± 4.10	13.35 ± 1.83	0.08 ± 0.01
9	E	78.90 ± 4.51	78.70 ± 4.50	56.31 ± 3.16	21.85 ± 1.59	0.14 ± 0.01
	C	64.36 ± 6.97	64.15 ± 6.94	46.07 ± 4.99	17.68 ± 2.01	0.15 ± 0.02
10	E	56.04 ± 6.99	55.86 ± 6.96	41.40 ± 5.05	14.06 ± 1.93	0.11 ± 0.02
	C	44.64 ± 4.80	44.54 ± 4.78	32.80 ± 3.57	11.35 ± 1.20	0.07 ± 0.01
11	E	54.26 ± 4.51	54.13 ± 4.50	40.03 ± 3.11	13.65 ± 1.43	0.08 ± 0.02
	C	46.55 ± 6.77	46.45 ± 6.75	34.39 ± 4.85	11.63 ± 1.86	0.07 ± 0.02
13	E	58.98 ± 6.19	58.82 ± 6.20	41.41 ± 4.12	17.09 ± 2.12	0.12 ± 0.02
	C	47.65 ± 5.18	47.50 ± 5.17	34.80 ± 3.61	12.43 ± 1.66	0.11 ± 0.01
				India		
4	E	25.04 ± 2.02	24.49 ± 2.03	18.15 ± 1.55	6.03 ± 0.70	0.46 ± 0.07
	C	20.50 ± 2.98	19.97 ± 2.96	15.83 ± 2.42	3.81 ± 0.52	0.41 ± 0.09
6	E	46.30 ± 5.44	46.07 ± 5.40	33.83 ± 3.97	11.41 ± 1.52	0.09 ± 0.01
	C	32.84 ± 2.93	32.71 ± 2.91	24.53 ± 2.15	7.70 ± 0.72	0.09 ± 0.01
7	E	43.72 ± 4.53	43.54 ± 4.52	34.45 ± 3.87	8.45 ± 1.01	0.09 ± 0.01
	C	45.45 ± 4.98	45.28 ± 4.96	34.03 ± 3.77	10.59 ± 1.20	0.10 ± 0.02
8	E	50.68 ± 5.66	50.51 ± 5.67	37.81 ± 4.30	12.11 ± 1.37	0.11 ± 0.03
	C	47.90 ± 5.71	47.75 ± 5.70	35.87 ± 4.26	11.19 ± 1.39	0.09 ± 0.01
9	E	39.43 ± 2.60	39.32 ± 2.59	29.58 ± 1.82	9.24 ± 0.83	0.08 ± 0.01
	C	30.17 ± 2.72	30.06 ± 2.71	22.87 ± 2.15	6.79 ± 0.54	0.06 ± 0.01
10	E	44.57 ± 6.85	44.42 ± 6.83	32.87 ± 5.24	11.03 ± 1.69	0.09 ± 0.01
	C	32.08 ± 4.67	31.91 ± 4.67	23.20 ± 3.55	8.35 ± 1.09	0.10 ± 0.01
11	E	32.36 ± 2.91	32.14 ± 2.90	23.08 ± 1.91	8.66 ± 0.95	0.18 ± 0.03
	C	21.10 ± 2.78	20.96 ± 2.77	15.54 ± 2.05	5.20 ± 0.70	0.12 ± 0.02
13	E	43.91 ± 3.73	43.69 ± 3.71	31.76 ± 2.68	11.45 ± 1.04	0.19 ± 0.03
	C	40.78 ± 3.05	40.61 ± 3.05	30.07 ± 2.22	9.93 ± 0.86	0.13 ± 0.02

Gluconasturtiin is the only aromatic glucosinolate in *B. juncea*. The glucosinolate profiles were similar in plants grown in both countries, despite differences in absolute levels (Table 2.1). Glucosinolate levels were significantly affected by herbivory and the numbers of days elapsed after damage (MANOVA; Table 2.2). Overall, glucosinolate levels were higher in damaged plants (Figure 2.1).

Table 2.2: MANOVA table testing for the effects of damage by *Spodoptera* spp. larval feeding on total, alkenyl, and indole glucosinolate concentrations, as well as trichome densities on the abaxial and adaxial surface of the third leaves of *Brassica juncea* plants grown in The Netherlands and India (n = 10)

	Source of variation	Pillai's trace value	F	Hypothesis d.f.	Error d.f.	P
		Glucosinolates				
The Netherlands	Day	0.591	5.045	21	432	<0.001
	Damage	0.172	9.857	3	142	<0.001
India	Day	0.960	9.550	21	426	<0.001
	Damage	0.088	4.508	3	140	0.005
		Trichomes				
The Netherlands	Day	0.285	3.732	8	180	<0.001
	Damage	0.109	5.436	2	89	0.006
India	Day	0.480	7.105	8	180	<0.001
	Damage	0.402	29.914	2	89	<0.001

ANOVA revealed that alkenyl glucosinolates were induced significantly in the plants in the Netherlands ($F_{1,144}$= 18.37, P<0.01) and in India ($F_{1,142}$ = 11.77, P<0.01), whereas indole glucosinolates were not significantly induced in plants grown in either country. Gluconapin and sinigrin, the two major alkenyl glucosinolates which together constitute 97% of total glucosinolates in *B. juncea*, were induced significantly in the plants grown in both countries (Table 2.1). As a consequence, total glucosinolates were also induced by herbivory in both the Netherlands ($F_{1,144}$= 28.26, P<0.01) and India ($F_{1,142}$ = 11.82, P<0.01).

The temporal induction pattern was comparable in the plants grown in both countries; glucosinolate levels in *B. juncea* were found to increase significantly within 4 days after damage and remained at higher levels with some fluctuations until the 10th day after damage (Figure 2.1).

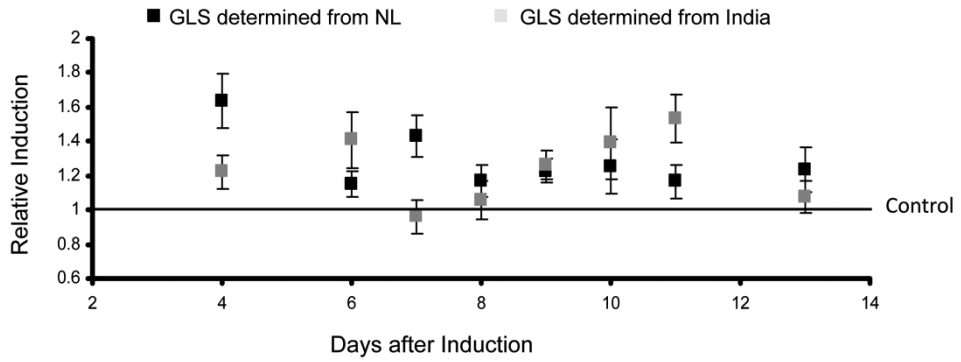

Figure 2.1: Relative induction of mean (± SE; n = 10) total glucosinolates in *Brassica juncea* leaves after induction by *Spodoptera* spp. in the Netherlands (NL) and India. Relative induction rate was calculated as (mean glucosinolates of damaged plants)/(mean glucosinolates of control plants). The horizontal line at value 1 indicates no induction.

At the end of our experiment, the systemically-induced glucosinolate response seemed to decline again. Amino acid and sugar levels showed no significant systemic response to herbivore treatment in the plants grown in either the Netherlands or India (Table 2.3).

Trichomes, leaf length, and leaf area

Our study showed that in *B. juncea* plants grown in either country, the trichome density was almost four times higher on the abaxial surface than on the adaxial surface of the third leaf (Table 2.4). Trichome density on both surfaces increased significantly due to herbivore damage (MANOVA, damage effect: $P<0.001$ in both countries; Table 2.2). The pattern of induction was found to be similar in the two countries: the strongest increase was seen from 7 to 11 days after induction. Maximum increase in the number of trichomes was seen on the 9th day after damage on both surfaces (Figure 2.2). Separate analysis of the surfaces showed that trichome density was significantly increased on the adaxial surface in the plants grown in India, but not in The Netherlands (ANOVA: $F_{1,90} = 12.92$ and 3.33, $P = 0.001$ and 0.071, respectively). On the other hand, on the abaxial surface, the numbers of trichomes increased considerably in plants grown in The Netherlands

Table 2.3: Mean (± SE; n = 10) of total sugars and total amino acids (nmoles mg)1) in experimental (E; damaged for 24 h by a *Spodoptera* spp. larva) and control (C) third leaves of *Brassica juncea* grown in The Netherlands and India. Days refer to number of days after insect damage

Days	Treatment	Total sugars		Total amino acids	
		The Netherlands	India	The Netherlands	India
4	E	142.35 ± 12.66	160.80 ± 18.90	368.35 ± 29.45	447.80 ± 43.42
	C	119.80 ± 16.60	172.61 ± 13.52	281.83 ± 35.05	448.93 ± 28.97
6	E	170.12 ± 22.89	275.87 ± 16.80	343.96 ± 37.09	780.69 ± 30.84
	C	208.94 ± 19.20	271.71 ± 21.36	345.34 ± 30.70	669.54 ± 43.35
7	E	145.72 ± 16.41	218.60 ± 11.42	286.94 ± 32.24	444.69 ± 19.69
	C	117.08 ± 12.71	249.54 ± 16.93	220.97 ± 15.61	484.62 ± 30.88
8	E	67.18 ± 6.90	372.26 ± 38.60	217.32 ± 10.96	470.39 ± 23.37
	C	77.38 ± 6.68	412.33 ± 30.13	239.39 ± 16.17	494.69 ± 29.29
9	E	71.54 ± 7.75	304.57 ± 20.77	275.14 ± 22.13	458.64 ± 18.70
	C	80.03 ± 10.08	267.74 ± 22.40	306.82 ± 29.02	379.41 ± 27.69
10	E	107.91 ± 7.21	288.52 ± 25.99	299.55 ± 18.52	399.41 ± 35.25
	C	120.21 ± 11.81	313.06 ± 23.48	296.42 ± 26.63	420.84 ± 19.87
11	E	164.58 ± 13.76	194.97 ± 15.74	359.05 ± 22.29	367.04 ± 19.97
	C	167.51 ± 20.93	196.23 ± 19.23	347.76 ± 45.06	397.80 ± 60.94
13	E	152.78 ± 10.41	216.57 ± 43.05	336.35 ± 22.52	320.80 ± 31.97
	C	152.25 ± 17.50	287.84 ± 42.31	329.86 ± 28.76	379.44 ± 39.57

Table 2.4: Mean (± SE; n = 10) density of trichomes on adaxial and abaxial surface of experimental (E; damaged for 24 h by a *Spodoptera* spp. larva) and control (C) third leaves of *Brassica juncea* grown in The Netherlands and India. Days refer to number of days following insect damage

Days	Treatment	The Netherlands		India	
		Adaxial	Abaxial	Adaxial	Abaxial
4	E	3.8 ± 0.7	20.7 ± 2.0	1.7 ± 0.1	7.9 ± 0.4
	C	3.2 ± 0.8	17.1 ± 1.8	1.3 ± 0.3	7.0 ± 0.3
7	E	4.7 ± 0.5	20.1 ± 1.7	1.2 ± 0.3	7.1 ± 0.2
	C	4.4 ± 0.4	19.6 ± 0.9	0.7 ± 0.3	5.5 ± 0.2
9	E	2.5 ± 0.3	20.8 ± 1.0	0.8 ± 0.3	7.8 ± 0.4
	C	1.4 ± 0.6	15.1 ± 2.2	0.4 ± 0.2	5.9 ± 0.4
11	E	2.3 ± 0.6	19.6 ± 1.5	2.4 ± 0.4	8.3 ± 0.3
	C	2.2 ± 0.6	15.7 ± 2.1	1.4 ± 0.3	6.6 ± 0.2
13	E	3.2 ± 0.7	19.5 ± 1.3	2.8 ± 0.5	9.4 ± 0.4
	C	2.1 ± 0.4	15.2 ± 2.0	1.7 ± 0.3	7.4 ± 0.4

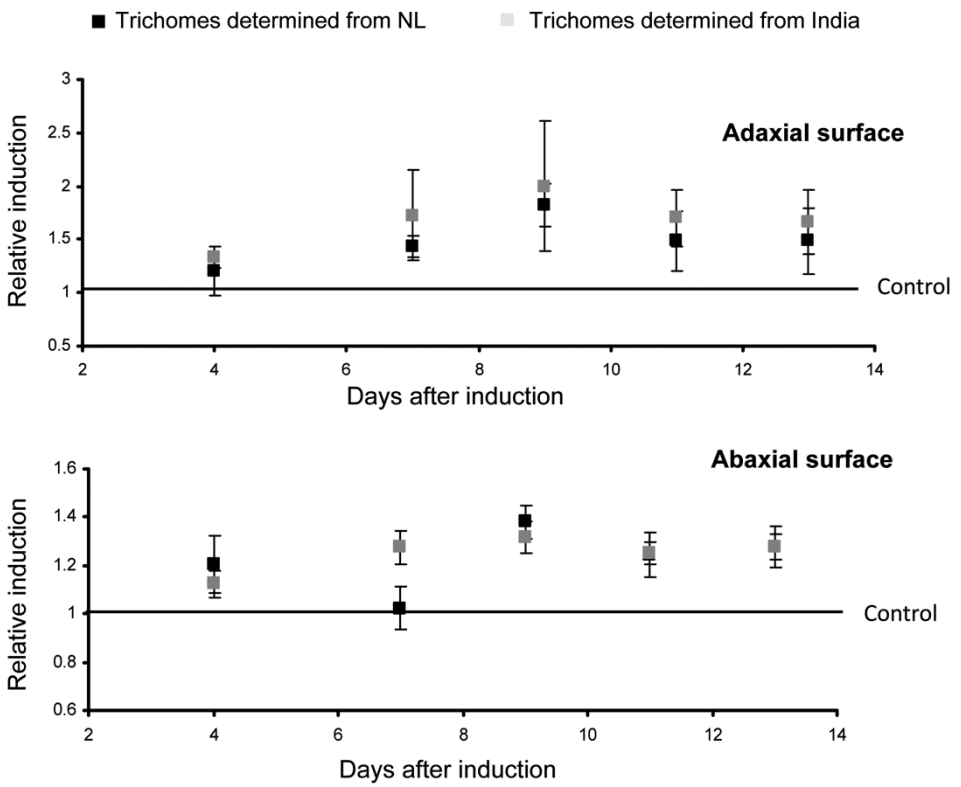

Figure 2.2: Relative induction of mean (± SE; n = 10) trichome density on the abaxial and adaxial surfaces of *Brassica juncea* leaves after induction by *Spodoptera* spp. in The Netherlands and India. Relative induction was calculated as (mean trichome density of damaged plants)/(mean trichome density of control plants). The horizontal line at value 1 indicates no induction.

as well as India ($F_{1,90}$ = 10.78 and 57.40, both P<0.001), compared to undamaged plants. Leaf length and area were not affected by damage, thus ruling out the possibility that increase in trichome density is because of leaf contraction or decreased leaf growth. Unlike glucosinolate levels, trichome densities did not decrease to control values after 13 days.

Feeding preference assays

Both third and sixth instar *S. litura* preferred leaves from undamaged to those from damaged plants (Table 2.5). In contrast, *P. xylostella* significantly preferred

leaves from plants damaged 9 days before, followed by those at day 4 after damage, whereas the control treatment was least preferred by these larvae (Table 2.5). The feeding preference between leaves taken at two time points after damage did not differ significantly for any of the larvae studied.

Table 2.5: Mean (± SE) amount of *Brassica juncea* leaf eaten (cm2) by third and sixth instar *Spodoptera litura* and fourth instar *Plutella xylostella* in 24 h, on leaves from undamaged plants (control) or damaged 4 or 9 days before (experiment)

	Instar	n	Control	Experiment		χ^2
				4th day	9th day	
S. litura	3	20	1.84 ± 0.17	0.38 ± 0.09	0.59 ± 0.13	21.90
	6	22	6.07 ± 0.54	2.50 ± 0.43	1.62 ± 0.32	30.78
P. xylostella	4	15	0.46 ± 0.07	0.60 ± 0.08	1.09 ± 0.09	16.30

χ^2 Friedman test: P<0.05

Insect performance bioassays

Nutritional indices indicated that the performance of *S. litura* larvae was significantly decreased when fed on herbivore-damaged plants (Kruskal-Wallis test: P<0.05). Larvae gained half the weight on leaves from damaged plants than larvae feeding on leaves from undamaged plants. This can be attributed to a significantly higher consumption (CI) of leaves on undamaged plants (Kruskal-Wallis test: P<0.05; Table 2.6). The values for AD, ECD, and ECI followed similar patterns, although the difference between treatment groups was not significant. Among leaves from damaged plants differences in AD, ECD, and ECI values were not significant (Table 2.6).

Table 2.6: Mean (± SE; n = 15) nutritional indices of sixth instar *Spodoptera litura* feeding on leaves from undamaged plants or damaged 4 or 9 days before

	Control	Experiment		P
		4th day	9th day	
Larval weight gain (mg)	0.16 ± 0.02	0.08 ± 0.02	0.05 ± 0.02	0.001
Consumption index (mg mg^{-1} day^{-1})	1.57 ± 0.21	0.68 ± 0.23	1.36 ± 0.45	0.026
Approximate digestibility (%)	51.31 ± 12.67	25.75 ± 14.29	29.01 ± 9.69	0.092
Efficiency of conversion of digested food (%)	182.15 ± 83.31	20.84 ± 62.16	73.63 ± 26.87	0.15
Efficiency of conversion of ingested food (%)	47.79 ± 21.04	69.86 ± 30.02	39.51 ± 11.35	0.77

Kruskal-Wallis ANOVA: d.f. = 2

Discussion

We found that both chemical and morphological responses of *B. juncea* were systemically induced as early as 4-7 days after damage by *Spodoptera* spp. In the two laboratories in which these responses were examined, the traits were induced to a similar extent and at the same temporal scale, despite differences in environmental conditions and the insect species that had been used to induce the plant. Glucosinolates, which are the key secondary chemical components in *B. juncea*, increased from day 4 onwards, remaining induced when compared to controls for at least 11 days after herbivore damage. Our study also showed a rapid and stable increase in trichome densities within 7 days. The systemic induction of glucosinolates was only slightly faster than that of trichomes. These results thus contradict our hypothesis that induction of structural defences in plants is slower than chemical induction. Whereas glucosinolate levels showed a tendency to subside to control levels at 2 weeks after damage, trichome densities remained stable. This can be explained by the fact that structural responses cannot easily be catabolised after induction. The only way that trichome densities could have decreased was through an increase in leaf area, which was not observed in our experiment. Given that amino acids and sugars were not significantly induced after damage, induction of glucosinolates and trichome densities may play a significant role in affecting insect preference and performance. Nevertheless, it should be considered that *Brassica* plants may contain various other inducible defences, for example, phenylpropanoids, protease inhibitors, and terpenes that were not measured here but certainly affect insects and their associated parasitoids (Broekgaarden *et al.* 2007; Jansen *et al.* 2009; Dicke *et al.* 2009).

For insect preference and performance bioassays, we selected a time point at which only glucosinolates were induced (day 4), and a time point at which both responses were maximal (day 9). In a three-choice feeding preference experiment between uninduced plants and plants that were damaged 4 or 9 days earlier, *P. xylostella* showed a strong preference for plants that were damaged 9

days earlier. This specialist is known to be attracted to, and remain unaffected by, high levels of glucosinolates because of the enzyme glucosinolate sulfatase which detoxifies these compounds (Renwick, 2002; Ratzka *et al.* 2002). In contrast, both third and sixth instars of *S. litura* strongly preferred the uninduced plant. Performance assays on sixth instars confirmed that larval weight gain was highest on control plants. Our results are in accordance with Müller & Sieling (2006), who found that performance of the specialist *A. rosae* remains unaffected throughout its development by high glucosinolate or myrosinase levels of *B. juncea.* However, this does not preclude that myrosinase levels may be important for *P. xylostella* resistance. Earlier, Li *et al*. (2000) found that the area damaged by *P. xylostella* and *S. eridania* was significantly affected by glucosinolate concentrations and myrosinase activities in 14 lines of *B. juncea*. Similar studies by Wallace & Eigenbrode (2002) provided evidence that the glucosinolate-myrosinase system in *B. juncea* cotyledons contributes to defence against *S. eridania*. In our study we did not analyse myrosinase levels in induced plants and future work in this direction could provide a better insight into the effects of the formation of glucosinolate conversion products.

Apparently, the elevated trichome density on day 9 did not have any effect on the feeding preference of *P. xylostella*, whereas *S. litura* may have been negatively affected by both glucosinolates and trichomes. Trichomes are considered as 'soft' weapons for insect defence as compared to other, lethal defences (Dalin *et al.* 2008). Earlier studies have established the role of trichomes in the resistance of both generalist and specialist herbivores (Agrawal, 1999, 2004; Traw & Dawson, 2002b; Clauss *et al.* 2006). *Plutella xylostella* remained unaffected by these trichomes, perhaps because of the small size of the larvae. In contrast, *S. litura* preferred least to feed on 'day 9' leaves which not only had higher glucosinolate concentrations, but also higher trichome densities. The combined effect of these responses was more evident in sixth instar preference, possibly because these larvae were too big to avoid eating trichomes on the leaf (Table 2.5). Trichomes are often composed of cellulose and other substances that have low nutritional value

(Levin, 1973). Therefore, when insects feed through the trichomes while feeding on the leaf epidermis, they gain less weight despite consuming more leaf (Dalin *et al.* 2008). This was evident from our performance experiment on sixth instar *S. litura* wherein we found that larvae gained less weight despite consuming larger amounts of leaves from plants induced 9 days earlier (Table 2.6).

Our study thus highlights the complexity of plant-herbivore interactions in nature. Each type of induced response has its own temporal dynamics and herbivores react individually to these responses. Thus, examining temporal dynamics and coordination of various induced plant-responses is essential, especially when studying insect resistance and its effect on higher trophic levels (Travers-Martin & Müller, 2008). Our results therefore contribute both to basic research concerning the physiology, ecology, and evolution of defence mechanisms of wild plant species, as well as to applied research devoted to the development of agricultural tools for crop protection. In addition, for an enhanced perspective on plant responses in nature, future research should involve the effect of induced responses on the dynamics of insect resistance and third-trophic-level parasitoids and predators whose activities may also affect plant fitness.

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Chapter 3

Dealing with double trouble: Consequences of single and double herbivory in *Brassica juncea*

Picture by: Vartika Mathur

Vartika Mathur, Tom O. G. Tytgat, Rob M. de Graaf, Vinay Kalia, A. Sankara Reddy, Louise E. M. Vet and Nicole M. van Dam

Submitted in a slightly modified version

Abstract

In their natural environment, plants are often attacked simultaneously by more than one species of insect herbivore. Induced plant responses to multiple attackers may differ from those after damage by a single species and this may influence the community of later arriving herbivores. Hence, under double herbivory, the specificity of responses that is often reported in studies with single herbivores may be compromised. The present study focuses on how single and double herbivory affects induced responses in plants, and whether these responses have divergent effects on successive herbivores at different time points after induction. Morphological (leaf length, leaf area and trichome density) and chemical changes (leaf alkenyl and indole glucosinolate levels) in *Brassica juncea* were evaluated 4, 10, 14 and 20 days after damage by the specialist *Plutella xylostella* alone, or together with the generalist *Spodoptera litura*. Preference and performance of both herbivores were tested on these time points to assess the biological effect of the plant's responses. We found that alkenyl glucosinolates were induced twenty days after damage by *P. xylostella* alone, whereas their levels were elevated as early as four days after double herbivory. Increases in trichome density were observed in both treatments, but they were stronger when damaged by the two herbivores simultaneously. Interestingly, there was an overall decrease in indole glucosinolates and an increase in leaf size due to damage by *P. xylostella*, which was not observed during simultaneous damage. In the behavioural assays, *S. litura* always preferred and performed better on undamaged plants. In contrast, *P. xylostella* always preferred damaged plants and performed better on plants damaged 14 days after damage by its conspecific and 10 days after double herbivory. Our results suggest that temporal studies involving single-versus multiple-attacker situations are necessary to fully comprehend the ecological and evolutionary aspects of plant-herbivore interactions in complex environments.

Introduction

Plants have to deal with various biotic and abiotic stresses in their surroundings and balance their resources to optimize growth, reproduction and defences. Insect herbivory often serves as a significant stress factor, and plants have evolved many different forms of defensive strategies to prevent it (Schoonhoven *et al.* 1998). Morphological structures on the plant such as trichomes, hairs, spines, and waxes can physically prevent insect herbivory. Plants also produce a number of secondary chemicals, which make their tissues unpalatable or toxic to herbivores, thus reducing herbivore damage (Schoonhoven *et al.* 2005). In addition to constitutive defences, which are always expressed, plants respond to herbivore attack by altering their defence levels. These changes are known as induced responses (Karban and Baldwin 1997). The effects of these responses on insect-plant interactions can differ greatly, and depend on many factors such as their concentration at the time of attack or whether the feeding herbivore is a generalist or a specialist on the plant. The advantage of induced over constitutive responses is that they are expressed only when necessary and can be specifically tailored to the herbivore that is attacking the plant (Karban *et al.* 1999).

The timing of induced changes determines which herbivores are affected. Each response may have its own temporal dynamics, which can be rapid or delayed. When there is a rapid response, the organism that causes the damage may suffer the consequences itself (De Moraes *et al.* 2001, van Dam *et al.* 2001). However, it is also possible that taxonomically unrelated species may bear the consequences when the response is delayed (Agrawal and Sherriffs 2001, van Dam *et al.* 2003, van Dam *et al.* 2004, Viswanathan *et al.* 2005). The effect of an induced response must also be considered in terms of the mobility and life history of the visiting herbivores. Mobile attackers are less affected than the sedentary ones, especially by local responses. Similarly, delayed responses may also influence the attacker itself when the herbivore has a long enough life cycle, or when the species has subsequent short-lived generations (Karban and Baldwin 1997).

An important trait of induced responses is their specificity. Different herbivores can induce different phenotypic responses in the plant that may either increase resistance or susceptibility to subsequent attack (Feeny 1976, Karban and Baldwin 1997, Van Zandt and Agrawal 2004b). Various studies suggest that plants adjust their responses according to specific attackers by regulating their signalling pathways in order to optimize their effectiveness (Pozo *et al.* 2004, Beckers and Spoel 2006, Leon-Reyes *et al.* 2009). Particular qualities of the damage, such as feeding strategy, salivary constituents of the damaging insect, amount of leaf eaten, and/or timing and pattern of damage may be perceived differently by the plant (Bowers and Stamp 1993, Mattiacci *et al.* 1995, Alborn *et al.* 1997, Walling 2000). Additionally, within a feeding guild, different species of herbivores may elicit different plant responses (Agrawal and Karban 1999, Voelckel and Baldwin 2004). The specificity of these responses may even depend on the plant species and the number of herbivore species damaging the plant. For example, de Boer *et al.* (2008) found that lima bean plants were more strongly induced due to damage by two herbivore species as compared to when attacked by a single herbivore, while some responses in cucumber were suppressed due to double herbivory.

In general, plant responses to more than one attacker may have three possible effects: 1) an additive response because of the lack of response specificity to different; 2) specificity in the plant's response with no trade-offs, whereby the plant response is different for each damaging herbivore and the plant induces a full response to each herbivore when it is damaged simultaneously by them; 3) specificity in the plant's response with trade-offs, in which the plant response is different for each damaging herbivore and becomes sub-optimal when two herbivores attack at the same time (Rodriguez-Saona *et al.* 2010). Moreover, specificity of the induced responses should be distinguished from specificity of the effect of the responses. These may not be similar, since one induced compound can have various effects on many different herbivores, while different compounds can have similar effects (Karban and Baldwin 1997, Agrawal 2000). For example, specialist and generalist herbivores may be differentially affected by the same

plant responses. Induced compounds that inhibit feeding by generalists may be used by specialists as feeding stimulants (Agrawal and Karban 1999). Thus, early herbivores inflicting damage on the plant play an important part in structuring the herbivore community that colonizes the plant later (Thaler *et al.* 1999, Van Zandt and Agrawal 2004a, Van Zandt and Agrawal 2004b, Poelman *et al.* 2009, Poelman *et al.* 2011). Therefore, induced responses are an important link between induced phenotypes and herbivore community composition.

In order to address the specificity of induced plant responses under single and double herbivory, we studied the effect *Plutella xylostella* damage alone, as well as of simultaneous damage by this specialist and the generalist *Spodoptera litura* on the induction of *Brassica juncea* resistance. We also determined the differences in behavioural responses and subsequent feeding by these two herbivores on either single or double infested plants.

B. juncea, commonly called Brown or Indian mustard is an annual herb of the family Brassicaceae and is cultivated in India and its neighbouring countries as a main source of mustard oil. In India, the seeds of this plant are sown between October and November and the harvesting of the seeds is done from February to March. This plant possesses trichomes as a constitutive structural defence (Mathur *et al.* 2011). Trichomes are known to affect the feeding preference and performance of insects at various stages of their lives as they physically impede the movement of insects (Fernandes 1994, Traw and Dawson 2002b, Agrawal and Fishbein 2006). Trichomes are known to systemically increase in density or number following insect damage in different species of Brassicaceae (Agrawal 1999, Traw 2002, Traw and Dawson 2002a). In addition, Brassicaceous plants, including *B. juncea*, are characterized by a class of secondary compounds called glucosinolates that are hydrolysed by myrosinases upon tissue damage, resulting in the formation of toxic products such as thiocyanates, isothiocyanates, epithionitriles and nitriles (Grubb and Abel 2006, Halkier and Gershenzon 2006, Hopkins *et al.* 2009). These induced secondary metabolites are known to have a negative influence on herbivores and play an important role in preventing further damage to the plants (van Dam and Raaijmakers 2006, van Dam and Oomen 2008, Hopkins *et al*. 2009).

For the induction experiments, we used the specialist insect *Plutella xylostella*, which feeds on virtually all species of Brassicaceae including *B. juncea* (Talekar *et al.* 1985, Talekar and Shelton 1993). Additionally, we have used *Spodoptera litura*, which is a generalist herbivore. Its larvae defoliate many economically important crops of different families such as Solanaceae, Poaceae and Brassicaceae (Brown and Dewhurst 1975). *S. litura* is one of the earlier infesting herbivores in *B. juncea* cultures in India. Both these insects have several generations in a year in India and can be found simultaneously on *B. juncea* due to overlapping life cycles.

With this study system, we aim to assess the temporal dynamics of specialist and double herbivory on induced plant responses, and their effect on subsequent feeding herbivores. Our main hypotheses are (1) induced responses are specific for each herbivore species damaging the plant, (2) induced plant responses are enhanced when the plant is damaged by more than one herbivore, (3) plants damaged by single and double herbivory have differential effects on the performance and preference of generalist and specialist herbivores.

Materials and methods

The experiments were conducted in Sri Venkateswara College, Delhi, India. For glucosinolates analyses, plants were grown in India, whereas the extraction and analysis was performed at Radboud University, Nijmegen, the Netherlands (NL).

Plants

Seeds of *B. juncea* var. *varuna* were obtained from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India, and stored dry in the dark at 10°C. The experiments were conducted from October until the beginning of January, which is the natural planting season of mustard crops in India. The plants were grown in an insect-free enclosure in garden soil in earthen pots. They were treated with 0.5 Hoagland solution (Hoagland and Arnon 1950) weekly for the first 2 weeks and twice a week from the third week onwards. The concentration of potassium phosphate (KH_2PO_4) in the Hoagland solution was doubled in the third

week and tripled from the fourth week to avoid phosphorus deficiency. Every alternate day, five randomly chosen pots were weighed to determine the volume of solution needed to maintain the water content in the pots at 14% of the soil dry mass.

Insects

Larvae of *S. litura* were obtained from laboratory cultures maintained at Sri Venkateswara College, New Delhi since 2008 on castor bean leaves and periodically supplemented with individuals from the Division of Entomology, IARI (New Delhi) to avoid inbreeding. Larvae of *P. xylostella* were obtained from the Division of Entomology, IARI, and were used directly in the experiment.

Induction of plants

All the experiments were performed when the plants were approximately four weeks old and in stage 63 according to BBCH scale (Lancashire *et al.* 1991). For specialist induction, two fourth instar *P. xylostella* larvae were allowed to feed on the third leaf from the apex of the plant. The larvae were placed on the leaf in a clip cage. For our experiments on simultaneous damage by a generalist and a specialist herbivore, we avoided direct contact between the two species by confining the generalist and specialist on two different leaves on the plant. Hence, two fourth instar *P. xylostella* larvae were placed on the third leaf and one third instar *S. litura* larva was introduced on the fourth leaf from the apex of the plant in a clip cage. The larvae were allowed to feed for 24 h. Undamaged plants received empty clip cages. After 24 h, the larvae and clip cages were removed. Damaged and undamaged plants were placed randomly on the tables.

For measuring systemic plant responses, the second leaf from the apex of damaged and control plants was harvested at 4, 10, 14 and 20 days after introduction of the larva. We started the period of our studies on day 4 because previous studies suggested that glucosinolate induction starts from the 3rd day after herbivore damage onwards (Hopkins *et al*. 2009, Mathur *et al*. 2011). Measurement of chemical and morphological responses were performed on

separate sets of plants with equal (n = 10) number of experimental and control plants for each day of the experiment.

Glucosinolate analysis

Harvested leaves were immediately frozen and stored at -20 °C. They were freeze-dried in India and sent to NL for extraction and High Performance Liquid Chromatography (HPLC) analysis. In NL, leaf samples were ground with a ball mill [Retsch ball mill (type MM301)]. 50-55 mg of finely ground plant material was extracted in 1.0 ml 70% methanol (MeOH) in water (v/v) in Eppendorf tubes, vortexed and immediately boiled at 90°C for five minutes to stop remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 minutes and centrifuged at 4,500 r.p.m. for 10 minutes. The supernatant was transferred to another Eppendorf tube. The extraction was repeated with the pellet once more as described above except the boiling step. Both supernatants were combined per sample in a corresponding clean and labelled 2 ml Eppendorf tube.

The supernatant was added to a DEAE-Sephadex A-25 column (5x10mm) and washed twice with 1 ml 70% MeOH, once with 1 ml MilliQ water and then twice with 1 ml 20 mM NaOAC buffer (pH 5.5). 20 μL of aryl sulfatase (Sigma type H-1 of Helix pomatia) was then added to the columns and flushed down with 50 μL NaOAC buffer to break the sulfur bonds of glucosinolates. The columns were then covered with aluminium foil and incubated overnight at room temperature. Thereafter, desulfoglucosinolates were eluted twice from the columns with 0.75 ml MilliQ water, and freeze-dried. The residue was dissolved in 1.0 ml of MilliQ water and stored at -20°C until further analysis.

Glucosinolate analysis was done according to van Dam *et al*. (2004). The extract was desulfated with arylsulfatase (Sigma, St. Louis, IL, USA) and separated on HPLC by means of a reversed phase C-18 column with a CH_3CN–H_2O gradient. Analysis was performed using a photodiode array detector with 229 nm as the integration wavelength (Dionex, Sunnyvale, CA, USA). Desulfoglucosinolate peaks were identified by comparison of retention times and UV spectra with a certified rape

seed standard (Community Bureau of Reference, Brussels, code BCR-367R) and authentic standards (progoitrin, gluconapin, glucoiberin, glucobrassicanapin, glucotropeaolin, gluconasturtiin, glucoraphanin, glucoerucin, glucobrassicin, sinalbin; Phytoplan, Heidelberg, Germany). To calculate glucosinolate concentrations in the plant tissue, the obtained values were divided by dry mass. Quantities of sinigrin, gluconapin and glucobrassicanapin were pooled together to obtain the total amount of alkenyl glucosinolates. Similarly, glucobrassicin and 4-methoxyglucobrassicin were combined to obtain total indole glucosinolates values.

Trichomes, leaf length and leaf area

Since trichomes were found only on the leaf veins, the number of trichomes on a 1 cm stretch was counted at four places on the adaxial as well as on abaxial surface using a dissecting microscope. The average number of trichomes per cm was calculated in each individual leaf. These leaves were then scanned using a Hewlett Packard flatbed scanner (Hewlett Packard Company, Palo Alto, CA, USA). The scanned images were used to measure the length and area of that leaf using the software 'ImageJ' with a global scale set to 106 pixels per cm (Rasband, 1997–2011; Abramoff *et al.*, 2004).

Insect behavioural and performance bioassays

Plants to be used on different time points after induction were induced on the respective days before the experiment, so that all the preference and performance experiments for each treatment group could start on the same day.

Feeding preference assays

A glass Petri dish of 20 cm diameter was lined with moist filter paper. Similarly sized leaves of undamaged plants and plants induced 4, 10, 14 and 20 days earlier were outlined with a pencil on a graph paper and placed at an equal distance from each other in a Petri dish. Feeding preference bioassays were conducted with either two fourth instar *P. xylostella* larvae or one sixth instar *S. litura* larva in one Petri dish (n = 10 per treatment). The larvae were introduced in the centre of the

Petri dish and allowed to move and feed freely between the five leaves. After 24 h, the larvae were removed and damaged portions of the leaves were outlined. The area damaged was then counted for each leaf and compared with each other.

Insect performance bioassays

For the performance experiment, the second leaf of the plants was removed, weighed and placed individually in a container lined with moist filter paper (n = 10 per treatment). Five containers were kept without larvae to determine leaf moisture loss. Newly moulted sixth instar *S. litura* or fourth instar *P. xylostella* larvae were starved for four hours. After weighing the larvae to the nearest 0.1 mg, they were introduced to the containers either with a leaf from a control plant or plants damaged 4, 10, 14 or 20 days earlier. The larvae, one *S. litura* and two *P. xylostella* per container, were allowed to feed on the leaf for 24 hours, after which they were weighed to calculate their weight gain. The unconsumed leaf was removed and weighed as well. The remaining leaf mass at the end of each experiment was subtracted from the initial mass of the leaf. An average change in mass due to moisture loss was determined from the leaves in containers without larvae and this was subtracted from the final mass of leaf remaining at the end of the experiment. Thus we obtained the actual mass of leaf ingested by the larva. The Consumption Index (CI) was calculated as [(leaf mass ingested)/ (larval mass gain x number of days)] and the Efficiency of Conversion of Ingested food (ECI) was calculated as [(larval mass gain)/ (leaf mass ingested)] (Waldbauer 1968).

Statistical analysis

The data was analysed using SPSS 17.0 (SPSSInc; Chicago, Illinois, USA). Normality and HOV were determined using 1-sample Kolmogorov-Smirnov test and Levene's test, respectively. For all the analyses, if the differences were found to be significant, Bonferroni post-hoc tests were conducted to correct for multiple comparisons.

The influence of 'treatment' and 'time after induction' on glucosinolates, trichome density and leaf size was analysed using MANOVA. The overall effect of damage was analysed using One-way ANOVA, and the relationship between

damage and their effect at different time points was compared using univariate ANOVA. The amount of leaf area consumed of each treatment was evaluated using a non-parametric Chi-square Friedman's test. The data on larval performance (weight of leaf consumed, larval weight, consumption index and efficiency of conversion of ingested food) were analysed using One-way ANOVA when the data met the assumptions of parametric analysis. When the assumptions of parametric analysis were not met, a non-parametric Kruskal-Wallis ANOVA was used.

Results

Glucosinolates

Glucosinolate levels were significantly affected by damage due to herbivory by the specialist *P. xylostella* alone as well as by simultaneous damage by *P. xylostella* and *S. litura* (MANOVA, Damage effect; Table 3.1).

Table 3.1: MANOVA table testing for the effects of damage by herbivores on the alkenyl and indole glucosinolates (μg/dry mass), trichome densities (per sq. cm) of the abaxial and adaxial surface and leaf length and area of the second leaf of *Brassica juncea* plants (n=10). PD represents the damage by the specialist *Plutella xylostella*; DD represents the double damage by *P. xylostella* and the generalist *Spodoptera litura* together.

	Source of Variation	Pillai's Trace Value	F	Hypothesis d.f.	Error d.f.	P
			Glucosinolates			
PD	Day	0.441	6.514	6	138	< 0.001
	Damage	0.166	6.781	2	68	0.002
DD	Day	0.045	0.738	6	136	0.795
	Damage	0.540	26.560	2	67	< 0.001
			Trichomes density			
PD	Day	0.638	11.230	6	144	< 0.001
	Damage	0.328	17.347	2	71	< 0.001
DD	Day	0.254	3.491	6	144	0.003
	Damage	0.497	35.044	2	71	< 0.001
			Leaf size			
PD	Day	0.622	10.821	6	144	< 0.001
	Damage	0.123	4.967	2	71	0.01
DD	Day	0.311	4.415	6	144	< 0.001
	Damage	0.259	12.436	2	71	< 0.001

However, we found a significant day effect in glucosinolate levels only when the plants were damaged by *P. xylostella* alone, but not in case of double herbivory (MANOVA, Day effect; Table 3.1). Alkenyl glucosinolates, mainly gluconapin (~73%) and sinigrin (~23%), constituted about 97% of the total glucosinolates. They were significantly increased due to damage by both the specialist alone (ANOVA; $F_{1,69}$ = 9.609, P < 0.005) as well as by simultaneous herbivory by the generalist and the specialist ($F_{1,68}$ = 59.214, P < 0.001) (Table 3.2).

Table 3.2: Mean ± SE (n = 10) of alkenyl and indole glucosinolates, gluconapin and sinigrin of experimental (Damaged; 24 h) and control (Undamaged) second leaves of *Brassica juncea*. Plants were damaged either by the specialist *Plutella xylostella* (*Plutella* damage) or by *P. xylostella* and generalist *Spodoptera litura*

Days	Treatment	Alkenyl	Gluconapin	Sinigrin	Indole
		Plutella damage			
4	Undamaged	25.10 ± 4.17	18.98 ± 3.15	5.90 ± 0.10	0.73 ± 0.07
	Damaged	30.94 ± 2.66	23.40 ± 1.95	7.22 ± 0.70	0.67 ± 0.13
10	Undamaged	26.98 ± 2.29	20.38 ± 1.78	6.34 ± 0.50	1.06 ± 0.08
	Damaged	32.27 ± 5.82	24.66 ± 4.43	7.21 ± 1.34	0.91 ± 0.11
14	Undamaged	39.82 ± 5.14	29.51 ± 3.44	9.82 ± 1.66	0.86 ± 0.10
	Damaged	51.40 ± 5.70	37.02 ± 3.58	13.67 ± 2.12	0.74 ± 0.10
20	Undamaged	32.72 ± 6.79	23.73 ± 4.94	8.63 ± 1.78	0.59 ± 0.13
	Damaged	59. 90 ± 9.62	40.14 ± 5.47	14.16 ± 2.55	0.48 ± 0.07
		Double damage			
4	Undamaged	28.29 ± 3.93	21.45 ± 3.05	6.57 ± 0.94	0.89 ± 0.14
	Damaged	55.73 ± 6.56	39.86 ± 4.52	14.94 ± 1.98	0.52 ± 0.07
10	Undamaged	25.10 ± 3.63	19.16 ± 2.94	5.63 ± 0.80	0.82 ± 0.19
	Damaged	67.62 ± 6.53	47.10 ± 4.10	19.35 ± 2.35	0.62 ± 0.07
14	Undamaged	35.85 ± 4.14	27.08 ± 2.97	8.33 ± 1.15	0.79 ± 0.08
	Damaged	49.37 ± 5.70	36.52 ± 3.86	12.02 ± 2.39	0.81 ± 0.12
20	Undamaged	20.87 ± 3.02	15.55 ± 2.27	5.17 ± 0.73	0.57 ± 0.08
	Damaged	57.93 ± 8.12	38.53 ± 5.01	18.26 ± 3.04	0.77 ± 0.14

Accordingly, total glucosinolate levels were also induced by the specialist ($F_{1,69}$ = 9.413, P < 0.005) and by the two herbivores together ($F_{1,68}$ = 58.128, P < 0.001) (Figure 3.1).

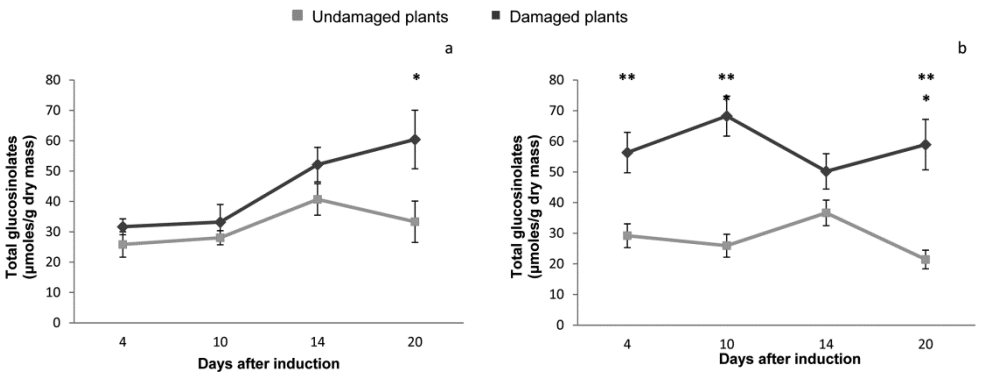

Figure 3.1: Total glucosinolates (µmoles/g dry mass) in the second leaf of *Brassica juncea* after systemic induction by (a) *Plutella xylostella* or (b) *P. xylostella* and *Spodoptera litura* together. Asterisks indicate significant induction (Univariate ANOVA; * = $P < 0.05$, ** = $P < 0.005$, *** = $P < 0.001$).

In contrast, indole glucosinolates differently responded to the two herbivore treatments. They decreased significantly due to specialist herbivory ($F_{1,69} = 2.330$, $P < 0.05$), but not after double herbivory ($F_{1,68} = 1.108$, $P = 0.296$) (Table 3.2).

Glucosinolate levels showed considerable differences in temporal patterns between the two herbivore treatments. After *P. xylostella* damage, glucosinolate levels began to increase to higher levels from the14th day onwards, but reached a significantly higher level only by the 20th day (Figure 3.1a). On the other hand, when plants were damaged by the two insects together, glucosinolate levels were significantly induced during most of the study period (Figure 3.1b).

Leaf trichomes, length and area

The density of trichomes on both adaxial and abaxial surfaces increased significantly due to damage by *P. xylostella* as well as by simultaneous damage by the specialist and the generalist (MANOVA, damage effect, $P < 0.001$ in both treatments; Table 3.1). Trichome density was found to significantly increase overall on both adaxial (1-way ANOVA; $F_{1,78} = 9.859$, $P = 0.002$) and abaxial ($F_{1,78} = 14.648$, $P < 0.001$) surfaces when plants were damaged by *P. xylostella*. This increase was significant except for the 14th day after damage (Figure 3.2 a, b).

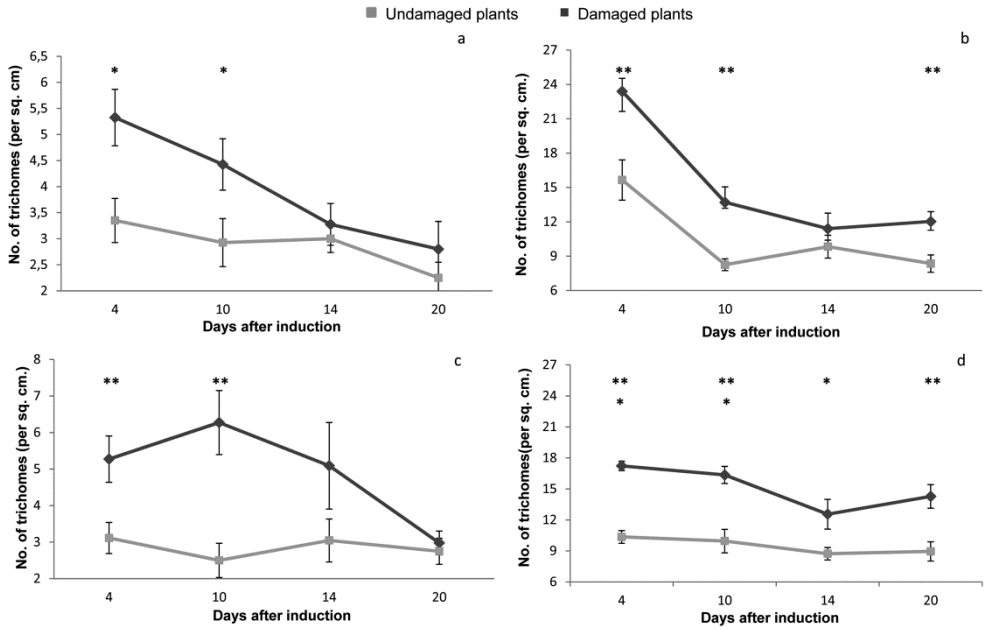

Figure 3.2: Trichome density after systemic induction by *Plutella xylostella* on the (a) adaxial and (b) abaxial surface and by *P. xylostella* and *Spodoptera litura* together on (c) adaxial and (d) abaxial surface of the second leaf of *Brassica juncea*. Asterisks indicate significant induction (Univariate ANOVA; * = $P < 0.05$, ** = $P < 0.005$, *** = $P < 0.001$).

Interestingly, we also found an overall increase in leaf length (1-way ANOVA; $F_{1,78}$= 5.122, P = 0.026) and area ($F_{1,78}$ = 4.792, P = 0.032). The strongest increase in length was seen on day 10 and in the leaf area on day 10 and 20 after the damage (Table 3.3).

Thus, due to damage by this specialist herbivore, plants not only increased the trichome density, but also the size of the leaf. When the plants were simultaneously damaged by *P. xylostella* and *S. litura*, overall trichome density was increased significantly on both adaxial (1-way ANOVA; $F_{1,78}$= 19.979, P < 0.001) and abaxial (1-way ANOVA; $F_{1,78}$ = 61.555, P < 0.001) surfaces. Separate analysis on individual days showed that this increase was the highest on day 4 and 10 after damage (Figure 3.2 c, d). Leaf length and area were not affected due to damage by these herbivores, thus ruling out the possibility that the increase in trichome density is due to leaf contraction or decreased leaf growth (Table 3.3).

Table 3.3: Mean (± SE; n = 10) leaf length and area of experimental (Damaged for 24 h) and control (Undamaged) second leaves of *Brassica juncea*. Plants were damaged either by the specialist *Plutella xylostella* (PD) or by *P. xylostella* and generalist *Spodoptera litura* together (DD). Days refer to number of days following insect damage. Asterisks indicate significant induction (Univariate ANOVA; $P < 0.05$).

Days	Treatment	Leaf length		Leaf area	
		PD	DD	PD	DD
4	Undamaged	7.23 ± 0.37	9.04 ± 0.28	28.78 ± 2.97	43.19 ± 2.66
	Damaged	7.38 ± 0.23	8.70 ± 0.37	26.77 ± 1.16	35.03 ± 3.19
10	Undamaged	8.81 ± 0.35	8.56 ± 0.48	39.86 ± 2.35	41.28 ± 4.70
	Damaged	10.00 ± 0.37*	9.99 ± 0.32*	48.84 ± 2.08*	47.81 ± 3.55
14	Undamaged	9.22 ± 0.33	9.26 ± 0.41	43.96 ± 2.65	49.67 ± 4.52
	Damaged	10.03 ± 0.24	9.07 ± 0.33	49.16 ± 3.65	38.11 ± 2.22*
20	Undamaged	8.99 ± 0.29	8.69 ± 0.43	35.68 ± 1.94	32.91 ± 2.75
	Damaged	9.58 ± 0.29	8.53 ± 0.28	44.67 ± 2.38*	33.19 ± 1.50

Feeding preference assays

When *P. xylostella* were offered a choice between leaves from undamaged plants or plants damaged by conspecific larvae at different time points, they significantly preferred leaves from plants damaged 10 and 14 days before, as compared to other treatments (Friedman test; $\chi^2(4) = 14.490$, $P = 0.006$). This preference shifted to leaves damaged 10, 14 or 20 days before when larvae were offered plants that were induced simultaneously by generalist and specialist herbivores ($\chi2(4) = 9.685$, $P = 0.046$) (Figure 3.3). In contrast, *S. litura* larvae consumed more leaf from the undamaged plants as compared to damaged plants, irrespective whether the plants were damaged by *P. xylostella* ($\chi2(4) = 21.737$, $P < 0.001$) or by the two herbivores together ($\chi2(4) = 13.843$, $P = 0.008$) (Figure 3.3).

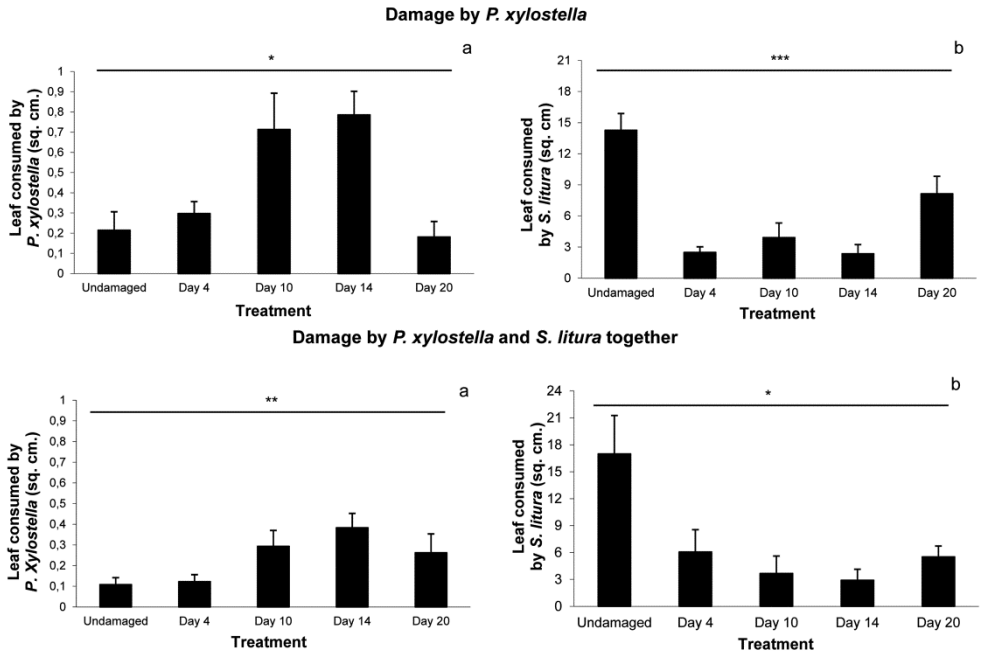

Figure 3.3: Mean (± SE) of amount of *Brassica juncea* leaf eaten (cm^2) by (a) fourth instar *Plutella xylostella* and (b) sixth instar *Spodoptera litura* when damaged by *P. xylostella* or by *P. xylostella* and *S. litura* together on leaves from undamaged plants or plants damaged 4, 10, 14 or 20 days before. Asterisks indicate significance level (Chi-square Friedman test; * = $P < 0.01$, ** = $P < 0.005$, *** = $P < 0.001$).

P. xylostella larvae fed significantly less on leaves damaged 4 days earlier by their conspecifics as compared to the other time points. However, this did not affect larval weight gain because the Efficiency of Conversion of Ingested food (ECI) was the highest for that time point. The ECI was significantly lower on leaves from undamaged plants, resulting in the lowest weight gain on these leaves. The larvae gained most weight when fed with leaves from plants damaged 14 days earlier. When larvae were fed with plants that were damaged by *S. litura* and *P. xylostella* together, their weight gain was the highest on plants damaged 10 days earlier and the lowest on undamaged plants (ANOVA with Bonferroni corrections; $P < 0.05$) (Table 3.4).

Table 3.4: Mean nutritional indices (± SE) of fourth instar *Plutellae xylostella* larvae (n=10). Plants were damaged either by the specialist *P. xylostella* (PD) or by *P. xylostella* and generalist *Spodoptera litura* together (DD). P-values were calculated using 1-way ANOVA for normal data and non-parametric Kruskal-Wallis ANOVA when the data was not normal.

	Treatment	Weight of the leaf consumed (mg)	Larval weight gain ± SE (mg)	Consumption Index± SE (mg/mg/day)	Efficiency of Conversion of Ingested food ± SE (%)
Undamaged	PD	112.65 ±7.85	1.18 ± 0.37	41.60± 11.48	0.06± 0.02
	DD	81.41 ± 14.24	1.72 ± 0.36	8.41 ± 2.58	2.08 ± 1.85
4- day damage	PD	27.90± 4.20	2.82± 0.51	1.97± 0.40	0.70± 0.13
	DD	75.11 ± 4.95	3.40 ± 0.64	3.34 ± 7.78	4.27 ± 6.73
10- day damage	PD	98.54± 3.00	3.86± 0.86	3.51± 0.55	0.39± 0.09
	DD	68.89 ± 10.97	7.42 ± 0.73	1.09 ± 2.73	1.52 ± 3.78
14- day damage	PD	101.98± 6.56	6.50± 0.97	2.92± 0.64	0.46± 0.07
	DD	32.00 ± 7.48	4.31 ± 0.75	8.83 ± 2.24	1.91 ± 5.52
20- day damage	PD	118.22± 8.00	4.00± 0.75	10.74± 4.64	0.20± 0.04
	DD	97.25 ± 7.15	2.60 ± 0.38	4.11 ± 3.70	2.67 ± 3.00
P- value	PD	$P < 0.001$ (ANOVA)	$P < 0.001$ (ANOVA)	$P < 0.001$ (Kruskal-Wallis)	$P < 0.001$ (ANOVA)
	DD	$P < 0.001$ (ANOVA)	$P < 0.001$ (ANOVA)	$P < 0.001$ (Kruskal-Wallis)	$P < 0.001$ (Kruskal-Wallis)

S. litura, on the other hand, gained the highest weight on leaves from undamaged plants as compared to plants damaged by *P. xylostella* at different time points. Their ECI was significantly lower on leaves from plants damaged 10 and 20 days earlier, on which they even lost weight. When fed on leaves from plants damaged simultaneously by the two herbivores, they consumed the least amount of leaf from plants damaged 14 days earlier. Interestingly, they consumed almost similar amounts of leaf of undamaged plants and those damaged 4 days earlier, but they showed the highest weight gain on plants damaged 4 days earlier. However, their weight significantly decreased when feeding on leaves from plants damaged 14 and 20 days earlier, as compared to others leaves, and also as compared to their weight at the start of the experiment (ANOVA with Bonferroni corrections; $P < 0.05$) (Table 3.5).

Table 3.5: Mean nutritional indices (± SE) of sixth instar *Spodoptera litura* larva (n=10). Plants were damaged either by the specialist *Plutella xylostella* (PD) or by *P. xylostella* and generalist *Spodoptera litura* together (DD). P-values were calculated using 1-way ANOVA for normal data and non-parametric Kruskal-Wallis ANOVA when the data was not normal.

	Treatment	Weight of the leaf consumed (mg)	Larval weight gain ± SE (mg)	Consumption Index± SE (mg/mg/day)	Efficiency of Conversion of Ingested food ± SE (%)
Undamaged	PD	193.64± 54.80	85.06± 21.45	4.92± 1.84	4.30± 1.73
	DD	249.44± 50.62	77.06± 16.44	6.65± 4.78	3.77± 1.18
4- day damage	PD	323.43± 89.61	26.20± 21.33	1.32± 1.06	8.88± 1.01
	DD	224.80± 32.29	96.29± 14.90	2.50± 2.25	4.32± 4.06
10- day damage	PD	92.26± 25.06	-33.55± 16.33	1.35± 1.20	-1.01± 4.23
	DD	184.94± 23.62	53.20± 12.81	1.82± 1.91	2.86± 5.23
14- day damage	PD	161.59± 98.61	1.54± 8.73	9.92± 3.48	2.65± 2.56
	DD	25.56± 16.20	-61.14± 12.29	-6.74± 3.55	-5.08± 2.63
20- day damage	PD	108.48± 28.05	-7.94± 21.32	3.43± 1.89	-4.51± 3.58
	DD	217.48± 40.06	-22.03± 31.13	1.61± 1.82	-3.21± 1.95
P- value	PD	$P = 0.018$ (Kruskal- Wallis)	$P < 0.001$ (ANOVA)	$P = 0.676$ (Kruskal- Wallis)	$P = 0.007$ (ANOVA)
	DD	$P < 0.001$ (ANOVA)	$P < 0.001$ (ANOVA)	$P = 0.014$ (Kruskal- Wallis)	$P < 0.001$ (Kruskal- Wallis)

Discussion

In nature, plants are often simultaneously exposed to more than one type of attacker. The primary objective of this study was to investigate whether plants respond differently in time when attacked by single or multiple herbivores, and whether these responses have divergent effects on further herbivory. When we measured changes in glucosinolate levels at 4, 10, 14 and 20 days after single or double herbivory, we found that alkenyl glucosinolates were increased as early as four days after double herbivory, but this induction was delayed until twenty days after damage by *P. xylostella* alone. Indole glucosinolates, on the other hand, significantly decreased following damage by the specialist but did not show any significant change after double herbivory. Trichomes were increased on both adaxial and abaxial surfaces following damage by single and double herbivory,

but the induction levels were higher when damaged by the two herbivores simultaneously. Interestingly, an overall increase in leaf size was found after damage by *P. xylostella*, but this was not observed after simultaneous damage by the generalist and specialist. In the behavioural bioassays, *S. litura* always preferred and performed better on undamaged plants, while *P. xylostella* preferred and performed better on plants that had been damaged at least ten days before.

In an earlier study, when plants were induced by the generalist *S. litura*, alkenyl glucosinolates increased significantly around 7 days after induction and remained at higher levels until 11 days after induction (Mathur *et al*. 2011). The present study revealed that alkenyl glucosinolate levels increased to significant levels only 20 days after damage by the specialist *P. xylostella.* There is ample evidence suggesting that induced plants are more attractive to this specialist (Reddy and Guerrero 2000, Poelman *et al.* 2008a, Sun *et al.* 2009). Therefore, delaying induced responses could be a plant strategy to escape further attack by not becoming more attractive. However, when damaged by *P. xylostella* and *S. litura* simultaneously, the increase in alkenyl glucosinolates was significant from 4 days after damage onwards. Glucosinolate levels in *B. juncea* thus increase more quickly in response to generalist feeding, either when they attacked individually or together with other herbivores. This is in accordance with many studies highlighting the role of secondary metabolite induction in deterring generalists (Kliebenstein *et al.* 2002, Long *et al.* 2007, Hansen *et al.* 2008). Interestingly, we found a significant overall decrease in indole glucosinolates when the plant was damaged by the specialist, which was not observed after damage by the generalist (Mathur *et al*. 2011) or by generalist and specialist together. Our study is in contrast with Poelman *et al.* (2008b) who observed an increase in indole glucosinolates in white cabbage cultivars due to damage by *P. xylostella*. This contrast may be explained by the differences in the defence strategies of different species within the same plant family.

Morphological attributes of plants also change in response to herbivory. In this study, we found increases in trichome densities on the adaxial and abaxial leaf surfaces after damage. We found a similar temporal pattern of trichome induction for both specialist and double herbivory on both surfaces. This temporal pattern is in accordance with earlier studies (Mathur *et al*. 2011). However, the induction in trichomes was stronger in case of double damage as compared to damage by specialist or generalist alone. To determine whether trichome density increased due to a decrease in leaf size, we also examined leaf length and area. Remarkably, we found a significant increase in both leaf length and area after damage by the specialist *P. xylostella*, which may indicate the activation of a tolerance mechanism by the plant after feeding by this specialist. This may partly explain the delay in glucosinolate induction as a trade-off mechanism, though further investigation is needed before drawing a firm conclusion in this respect. This increase was not observed when the plants were induced simultaneously by the specialist and the generalist, thus suggesting that specific responses to specialist feeding may be interrupted when a second, generalist, herbivore is feeding on the plant.

These different levels and patterns of responses to each treatment support earlier findings that plants have the ability to distinguish between different types of biotic challenges and respond specifically to each of them. Moreover, we found that when damaged by only their conspecifics, specialist *P. xylostella* preferred to feed the most and gained most weight on plants damaged 10 and 14 days earlier. They preferred and gained the least weight on undamaged plants. These results are in accordance with earlier studies that suggest that *P. xylostella* uses glucosinolates as cues to find its preferred host plant (Palaniswamy *et al.* 1986, Shiojiri *et al.* 2001, Sun *et al.* 2009). However, our results on specialist performance bioassays are in contrast with studies on wild radish by Agrawal (2000), who found that although *P. xylostella* was the least affected by induction due to other generalists and specialist insects, its growth was reduced by plants that were initially damaged by its conspecifics. *P. xylostella* is known to possess a

glucosinolate sulfatase enzyme that degrades glucosinolates in their host plants before the more toxic conversion products are formed (Ratzka *et al.* 2002). Nevertheless, it is possible that although glucosinolates have a stimulatory effect on these specialists, various other induced phytochemicals that we have not measured may act as deterrents to them (Agerbirk *et al.* 2003, Hodge *et al.* 2006).

On the other hand, *S. litura* always chose to feed on the undamaged plants as compared to plants damaged by either the specialist alone or together with a conspecific. When fed on plants damaged by the specialist alone, between 10 and 20 days earlier, these larvae even lost weight. In previous studies, *S. litura* also showed reduced preference and performance when the plants were damaged by its conspecific larvae (Mathur *et al.* 2011). This indicates that the damage-induced responses by a single attacker, irrespective of its species, are effective in conferring resistance against this generalist.

The importance of temporal dynamics in our induction studies was highlighted by the effect that double herbivory had on herbivore preference and performance. When *P. xylostella* were offered leaves from undamaged plants and plants damaged simultaneously by both specialist and generalist, they preferred to feed the most on plants damaged more than a week earlier, and the least on undamaged plants and plants damaged less than a week earlier. They performed significantly better on plants damaged 10 days earlier, when all the responses tested were the highest. Their performance was the lowest on leaves from undamaged plants, and also on plants damaged more than two weeks earlier. In contrast, *S. litura* larvae showed the highest larval weight gain on plants damaged 4 days earlier by the two herbivores, which was the first time point tested after damage in our studies. But they lost considerable weight when fed with plants damaged more than two weeks earlier. These results clearly demonstrate the different sensitivities of generalist and specialist herbivores to the temporal dynamics of induced plant responses. Generalists perform well, while specialists do not show a particular preference to the damaged plants after a short time lapse following herbivory when the induced responses are still not strong. Once

these responses are enhanced, the generalists avoid these plants, while the specialists prefer and perform better on these plants. Moreover, in our current study, the damaging herbivores were allowed to feed on the plant for only 24 hours and systemic leaves were offered to the experimental herbivores, supporting the notion that the plant acts as a mediator in this 'horizontal' interaction between initial and subsequent herbivores.

In India, the generalist *S. litura* and the specialist *P. xylostella* have multiple generations in a year, with overlapping host plants, such as *B. juncea*. This suggests that in fields, plants gain an enhanced resistance to *S. litura* through induced resistance mechanisms, but in the process, become more susceptible to *P. xylostella*. Thus, generalist pests such as *S. litura* can be combated by enhancing natural induced resistance in *B. juncea*, whereas other pest management measures, such as biological control through predators and parasitoids and cultural practices, such as crop rotation and trap cropping, should be applied in pertinence to specialists like *P. xylostella*.

Plant resistance mechanisms are thought to be evolutionary selected for when the attack is correlated with the risk of future attack and may be strongest when current and future attack is likely by the same organism (Karban *et al*. 1999). Although there are only a few previous studies on simultaneous herbivory, nevertheless they suggest that knowledge of single attacker systems may not predict the responses in a multiple-attack (Dicke *et al.* 2009). Moreover, most of the studies investigate these interactions only for a limited time period. The present study unravelled the intricacies behind these responses on a relevant temporal scale and demonstrated the complexity of these interactions when more than one attacker is involved. Thus it supports earlier finding that induced responses determine further species interactions, even when these species are spatially and temporally separated (Poelman *et al*. 2011). Therefore, temporal studies involving single- versus multiple-attacker situations are necessary to comprehend the evolution of induced defence strategies and the mechanisms behind plant-herbivore interactions in complex environments.

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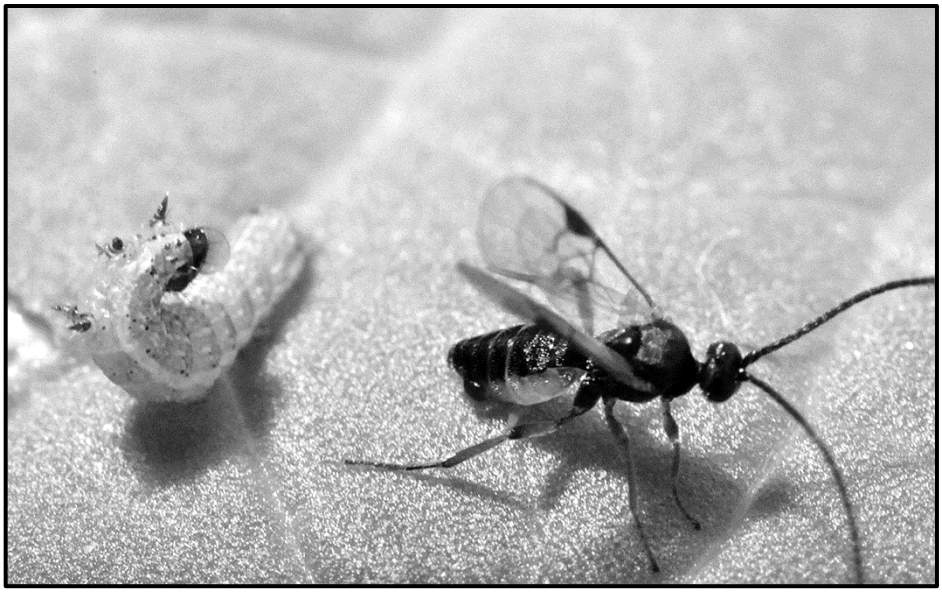
Chapter 4

Temporal dynamics of induced volatiles in *Brassica juncea*: chemical, molecular and ecological aspects



Picture by: Sonia Dourlot

Vartika Mathur, Tom O. G. Tytgat, Cornelis A. Hordijk, Harry R. Harhangi, Joroen J. Jansen, Jeffrey A. Harvey, A. Sankara Reddy, Louise E. M. Vet and Nicole M. van Dam

Abstract

Damaged plants of the family Brassicaceae emit volatiles upon herbivore damage, which have different effects on the associated insect community. They may act as repellents to generalist insect herbivores, whereas specialist insect herbivores and parasitoids can use these volatiles as cues to find their hosts. We investigated the temporal dynamics of induced volatiles after feeding by the larvae of generalist herbivores of *Spodoptera* spp. and their influence on subsequent herbivores and their parasitoids. The volatile blend of plants that had been damaged for 24, 48 or 72 hours mainly consisted of terpenoids, sulfides and green leaf volatiles, and showed a dynamic pattern over time. Gene expression analysis suggested that the induced response after *Spodoptera* feeding is mainly controlled by the jasmonic acid pathway in both damaged and systemic leaves. Several genes involved in sulfide and green leaf volatile synthesis were clearly upregulated. The effect of damage on gene expression levels was more pronounced in the local leaves as compared to the systemic leaves. The generalist *Spodoptera litura* preferred undamaged plants, whereas its parasitoid *Cotesia marginiventris* favoured plants damaged for 48 hours. The specialist *Plutella xylostella* and its parasitoid *C. plutellae* preferred plants damaged for 72 hours. This study thus shows that herbivore-induced plant volatile blends vary considerably over a short period of time, and elicits differential preferences in specialists and generalists insects in the associated food web. Therefore the temporal dynamics of herbivore-induced volatiles may play a key role in insect community composition after induction.

Introduction

Volatiles emitted by plants play an important role in mediating interactions with neighbouring plants, herbivores, carnivores, mutualists and microbes (van Dam 2009). Herbivore feeding may induce plants to emit volatiles in higher amounts or even trigger the synthesis of novel volatiles that affect the response of associated insects in different manners (Dicke and Vet 1999, Paré and Tumlinson 1999). On one hand, they attract or deter herbivores feeding on these plants (De Moraes *et al.* 2001, Halitschke *et al.* 2008), and on the other hand, they indirectly protect the plant from an attacking herbivore by facilitating host location by parasitoids and predators (Vet and Dicke 1992, Dicke and van Loon 2000, Poelman *et al.* 2008). Thus, herbivore-induced plant volatiles (HIPVs) act as a double-edged sword, defending the plants directly by deterring insects as well as indirectly by attracting predators and parasitoids of these herbivores.

The volatile blend emitted by plants is complex and chemically diverse. It may vary qualitatively or quantitatively following herbivore damage (Boland *et al.* 1999, Arimura *et al.* 2009, Dicke and Baldwin 2010). Natural enemies of herbivores rely on induced plants odours to locate their hosts or prey (Turlings *et al.* 1990, Dicke and van Loon 2000, Shiojiri *et al.* 2010). The reliability of HIPVs arises from the fact that plants release specific odour blends when damaged by herbivores and that utilizing these cues increases the chances of encountering suitable hosts (Vet and Dicke 1992, Turlings *et al.* 1993, Vet 1999). When HIPVs are qualitatively different from volatile blends of undamaged plants, the newly formed compounds may be taken as a cue for locating herbivore-infested plants (Holopainen 2004, Vuorinen *et al.* 2004, Gols *et al.* 2011). However, even when HIPV blends do not vary qualitatively, carnivores may still discriminate between undamaged and herbivore-damaged plants by changes in the quantity of certain volatile constituents in the blend (Dicke 1999).

HIPVs can essentially be categorized according to their biosynthetic pathways into three basic chemical classes: (i) Fatty acid-derivatives, called green leaf

volatiles (GLVs), which are C_6 aldehydes, alcohols and their derivatives, and jasmonic acid (JA), which is produced by the lipoxygenase (LOX) pathway. GLVs are produced via hydroperoxide lyase (HPL) pathway, which is a component of LOX pathway. (ii) Terpenoids, which are synthesized from mevalonate and non-mevalonate (also called methylerythritol phosphate or MEP) pathways. (iii) Volatiles derived from the shikimic acid pathway, such as indole and methyl salicylate. This pathway connects carbohydrate metabolism to the biosynthesis of aromatic compounds in plants (Paré and Tumlinson 1997, Holopainen 2004, Conti *et al.* 2008). Once the basic skeleton of these small molecular weight compounds are produced via these pathways, their diversity is achieved by modifications such as acylation, methylation, oxidation/reduction and cyclic ring closure. Such modifications often result in increased volatility and changed olfactory properties (Pichersky *et al.* 2006). In general, more than one biochemical pathway is responsible for a blend of volatile compounds released following herbivory.

In Brassicaceae an additional class of volatiles is found. This plant family is characterized by the presence of glucosinolates. Upon herbivory, these secondary metabolites are hydrolysed by the enzyme myrosinase, which yields volatile products such as nitriles and (iso)thiocyanates, depending on the reaction conditions. These hydrolysis products are associated with a direct plant defence strategy against various insects and pathogens (Gols and Harvey 2009, Hopkins *et al.* 2009, van Dam *et al.* 2009, Mathur *et al.* 2011), but can also serve as cues to natural enemies (Geervliet *et al.* 1997, van Dam *et al.* 2003, Gols *et al.* 2008, Gols *et al*. 2011). Often, the hydrolysis products of glucosinolates are methylated to volatile sulfur compounds (Attieh *et al.* 2000). These, along with other volatiles of the above mentioned three classes contribute to the typical *Brassica* 'odour'.

Plants produce various phytohormones involved in the signalling of herbivore-induced responses. The phytohormones salicylic acid (SA), JA and ethylene (ET) are the main regulators involved in induced responses (Gatehouse 2002, Howe and Schaller 2008, Dicke and Baldwin 2010). Based on herbivore

derived external cues, such as salivary compounds and feeding patterns, a specific combination of these hormones is produced (De Vos *et al.* 2005, Ehlting *et al.* 2008). Cross talk between the signalling pathways helps the plant to fine-tune its defence response to the invaders encountered (Pieterse and Dicke 2007, Koornneef and Pieterse 2008, Verhage *et al.* 2010). The jasmonate family of plant signalling molecules plays a major role in the regulation of induced plant responses against herbivory (Koo *et al.* 2009, Koo and Howe 2009). ET and JA act synergistically, while SA is known to act antagonistically on the JA response (Adie *et al.* 2007, Koornneef and Pieterse 2008, Verhage *et al*. 2010). Therefore, signalling interactions can be either antagonistic or synergistic, which eventually determines the functional outcome.

One single herbivore may induce many different responses, and each response may have its own time course (Mathur *et al.* 2011). In general, HIPV responses are known to be dynamic and specific (De Moraes *et al.* 2001). The blend of volatiles quickly changes within a short time span of days or even hours. Recent studies have tried to unravel the process by which plants perceive insect attack and trigger defence responses such as induced volatile emissions (Arimura *et al.* 2005, De Vos *et al.* 2007, Soler *et al.* 2012). To date, very few studies have rigorously quantified the temporal changes of signalling pathways and plant defence responses during actual insect herbivory (Schmelz *et al.* 2003, Bruinsma *et al.* 2009). Therefore, we explored the temporal dynamics and ecological significance of HIPVs in Brown mustard, *Brassica juncea* (L.) Czernov, when damaged by generalist lepidopteran herbivores from the genus *Spodoptera.* We compared the quantitative and qualitative differences in HIPV production between a leaf damaged for 24, 48 and 72 h following herbivory by *S. exigua* and similar leaves from an undamaged plant.

Thereafter, we determined the changes in gene expression in the damaged and a systemic leaf to examine interactions between the hormonal pathways and resulting gene expression levels in volatile synthesis pathways. To investigate the

hormonal pathways involved, we measured the expression of two marker genes for the JA pathway (*MYC2* and *VSP2*) (Berger *et al.* 2002, Lorenzo *et al.* 2004, Dombrecht *et al.* 2007), one gene for SA pathway (*PR1*) (Bowling *et al.* 1997) and two genes for the ethylene pathway (*ETR1* and *ERF1*) (Lorenzo *et al.* 2003, Lorenzo, *et al.*. 2004). Additionally, we measured the gene expression of the most prominent volatile classes found in Brassicaceae. Among the terpenoids, we examined the gene expression of *TPS10* (Bohlmann *et al.* 2000) and *TPS21* (Tholl *et al.* 2005) which are involved in the synthesis of mono- and sesquiterpenes, respectively. Other than terpenes, we measured the expression of three GLV genes, *viz.* hydroperoxide lyase1 (*HPL1*) (Matsui *et al.* 1999, Matsui 2006), Chloroplastic Aldehyde Reductase (*Chl*ADR) (Yamauchi *et al.* 2011) and acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (*CHAT*) (D'Auria *et al.* 2007). Glucosinolates are an important induced defence in Brassicaceae, and are the precursors for several sulfur-containing volatiles such as isothiocyanates and nitriles. Therefore, we measured the expression for genes involved in the synthesis of aliphatic (*CYP79F1* and *CYP83A1*) (Bak and Feyereisen 2001, Chen *et al.* 2003, Naur *et al.* 2003) and indole glucosinolates (*CYP79B2*) (Glawischnig *et al.* 2004). We also measured the gene expression of thiol methyltransferase1 (*TMT1*), a gene involved in the production of sulfides, which is another group of sulfur-containing volatile compounds emitted by *Brassica* species (Attieh *et al.* 2002).

Finally, in order to determine the ecological impact of HIPV temporal dynamics in *B. juncea*, we examined the orientation preference of the generalist herbivore *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) and the specialist *Plutella xylostella* L. (Lepidoptera: Plutellidae) and their parasitoids *Cotesia marginiventris* Cresson and *C. plutellae* Kurdjumov (Hymenoptera: Braconidae) respectively, to undamaged plants and plants damaged 24, 48 and 72 h before. Together these molecular, chemical and ecological data will provide a comprehensive picture of the different temporal phases involved in the induction of HIPVs in *B. juncea*.

Materials and methods

Plants

Seeds of *B. juncea* var. *varuna* were obtained from the Division of Genetics, IARI, New Delhi, India in 2008 and stored dry and in the dark at 10°C. They were germinated on glass beads in water in 10x10 cm plastic containers with a clear plastic lid. In the Netherlands, the greenhouse was kept at 21°C during the day and 16°C at night, under ambient light conditions that were supplied by sodium lamps to maintain the minimum PAR at 225 μmoles $m^{-1}s^{-1}$ for at least 16 h. Seven days later, the seedlings were transferred to 1.8-L pots, containing 1000 g peat soil-sand mixture (Lentse Potgrond no. 4, Lent, the Netherlands). From the third week onwards, plants were supplied with 0.5 Hoagland solution (Hoagland and Arnon 1950) once a week.

In India the plants were grown in an insect-free enclosure from October until the beginning of December 2009, which is normally the mustard growing season in India. They were grown in garden soil in earthen pots and treated with 0.5 Hoagland solution weekly.

Insects

Egg batches of beet armyworm (*Spodoptera exigua* Hübner; Lepidoptera: Noctuidae) and pupae of the parasitoids *C. marginiventris* and *C. plutellae* were obtained from the Department of Entomology, Wageningen University, the Netherlands. They were maintained in a climate room at 27°C, 50-70% RH and a 16-h light/8-hr dark photoperiod. A culture of *S. exigua* was maintained on an artificial diet as described by (Vickerman and Trumble 1999). Unmated naïve females of parasitoids were used directly after emergence for the experiments.

Larvae of *S. litura* and *P. xylostella* were obtained from laboratory cultures maintained at Sri Venkateswara College, New Delhi, since 2008 on castor bean and cabbage leaves, respectively. The cultures were supplemented with individuals from the Department of Entomology, IARI (Pusa, New Delhi) to avoid inbreeding. Unmated naïve females of both herbivores were used for the experiments within 24 h of emergence.

Induction of plants

All the experiments were performed when the plants were approximately four weeks old and in stage 63 according to BBCH scale (Lancashire *et al.* 1991). A single fourth instar of *S. exigua* (in the Netherlands) or a third instar of *S. litura* larva (in India) was introduced in a clip cage to the fourth leaf counted from the apex of the plant. The larvae were allowed to feed for 24, 48 or 72 h, depending on the treatment group the plant was assigned to. Undamaged plants received empty clip cages. Damaged and undamaged plants were placed randomly on the tables.

Volatile analysis

The volatile collection was conducted over five consecutive days in the greenhouse at the Netherlands Institute of Ecology (NIOO-KNAW), Heteren, the Netherlands. Clip cages, along with the *S. exigua* larvae were removed prior to volatile sampling and immediately put back thereafter. Thus, experimental plants damaged for 24 h were used the next day as those that were damaged for 48 h, and the following day as those that were damaged for 72 h. The damaged plants, 12 biological replicates in total, were divided in three days and repeatedly measured at 24, 48 and 72 h after damage. The undamaged plants, 3 biological replicates in total, were repeatedly measured on all five days of the experiment and received empty clip cages (Supplementary figure 1).

Prior to the experiment, turkey roasting bags (Toppits, Melitta Nederlands BV, Gorinchem, The Netherlands), 25 X 40 cm were sterilized at 120°C for two hours in an oven (Stewart-Jones and Poppy 2006). Bags were individually placed around a single leaf that was subjected to larval treatment in damaged plants or a leaf of similar age on undamaged plants. The bag was fitted with a steel trap, placed just above the leaf. Volatiles were collected by pulling the headspace air with a vacuum pump over the trap filled with 150 mg Tenax TA and 150 mg Carbopack (Markes International Ltd., Llantrisant, UK). Flow rates over the traps

were set to 100 ml/min using mass flow regulators (Sho rate TM, Brooks Instrument, Hatfield, PA, USA). After 60 min, the traps were removed, capped and stored at 4°C until analysis. Four plants were sampled in parallel during the experiment, and each day one background volatiles profile from an empty bag was sampled during the course of the experiment. Volatiles were desorbed from the traps and analysed by GC-MS using the same method and reference compounds as described by van Dam *et al.* (2010). All integrated signals were generated from the MS chromatograms by the AMDIS software (NIST, USA). To correct for minor differences in sampling time and flow rates over individual traps, peak areas obtained in each sample were divided by the total volume in ml that was collected over the trap. Peaks related to mixtures of volatiles and impurities were removed from the dataset. Background volatiles were uniformly subtracted from the volatiles of both undamaged and damaged plants to obtain only volatiles emitted by the leaves. In order to remove the day to day variation on the five experimental days, for each day of sampling the logarithmic fold changes in emission of individual volatile compounds were calculated by the formula: ln [treatment (peak area+1)/control (peak area+1)]. Thereafter, the obtained fold changes for each sampling day were averaged (Table 4.1).

Real time Q-PCR analysis

Gene expression analysis was conducted at Radboud University, Nijmegen, the Netherlands. Because of the technical set-up in the previous experiments, where the volatiles measurement had been performed on the locally induced leaf and for the choice experiments the parasitoids had been exposed to the volatiles of the whole plant, we also investigated if there is any difference in the temporal expression of volatiles between local and systemic leaves. In addition, we compared gene expression between larval induced response and JA induced response.

Table 4.1: Mean ± SE of the fold changes of volatile emissions calculated as ln [treatment (X+1)/control (X+1)] per day of sampling (n=3). X = peak area of the compound. Values in bold represent significant increases in volatile emissions over controls (p-value<0.05 after Independent sample t-test following Rieu and Powers 2009)

No.	Compound name	LRI (RTX-5ms)*	Compound class	Mean ± SE fold changes ln(X+1) 24 h	Mean ± SE fold changes ln(X+1) 48 h	Mean ± SE fold changes ln(X+1) 72 h
1	1-butene-4-isothiocyanate	979	isothiocyanates	**4.33 ± 1.02**	4.72 ± 1.02	**6.54 ± 0.59**
2	2-beta pinene	971	monoterpene	-0.31 ± 0.15	0.33 ± 1.33	-0.84 ± 0.46
3	alpha-pinene	927	monoterpene	-0.57 ± 1.35	0.48 ± 1.45	-0.46 ± 1.17
4	carene isomer or pseudolimonene	1007	monoterpene	-1.73 ± 0.87	-0.89 ± 1.80	-2.00 ± 0.80
5	limonene	1026.5	monoterpene	0.51 ± 0.49	-0.38 ± 0.69	-0.67 ± 0.12
6	beta-ocimene	1048.8	monoterpene	-2.57 ± 1.87	-3.01 ± 1.73	-0.27 ± 1.52
7	alpha-copaene	1371	sesquiterpene	0.25 ± 0.76	-0.48 ± 0.66	0.30 ± 0.69
8	farnesene isomer (E,E, alpha farnesene)	1506.4	sesquiterpene	1.67 ± 0.94	0.67 ± 0.53	0.10 ± 0.10
9	(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)	1577	homoterpene	4.45 ± 1.30	2.00 ± 1.12	1.69 ± 1.03
10	<3E>-4,8-dimethyl-1,3,7-nonatriene (DMNT)	1117	homoterpene	3.10 ± 1.59	0.48 ± 0.48	**1.93 ± 0.41**
11	3-hexen-1-ol <E> or <Z>	851	GLV	**3.10 ± 1.58**	**4.22 ± 0.86**	4.89 ± 0.89
12	3-hexen-1-ol-acetate (mix of <E> and <Z>)	1008	GLV	**1.42 ± 0.43**	1.20 ± 0.19	**2.14 ± 0.38**
13	acetaldehyde	498	GLV	-0.77 ± 0.36	**3.50 ± 1.88**	0.97 ± 1.88
14	acetic acid ethyl ester	600	GLV	1.43 ± 1.26	0.35 ± 1.55	-0.56 ± 2.91
15	acetic acid hexyl ester	1015.6	GLV	0.66 ± 2.25	1.03 ± 1.03	3.12 ± 1.73
16	hexadecanoic acid methyl ester	1928.6	GLV	0.94 ± 0.62	0.31 ± 0.80	-0.94 ± 0.62
17	dimethyldisulfide	738.8	sulfide	0.33 ± 0.14	0.27 ± 0.29	**0.42 ± 0.18**
18	dimethyltrisulfide	961	sulfide	0.57 ± 1.43	2.35 ± 1.91	**2.60 ± 1.13**
19	salicylic acid hexyl ester	1673.5	SA	-0.33 ± 0.86	-1.39 ± 0.65	-1.98 ± 0.29
20	1-dodecanol	1474	alcohol	-1.84 ± 1.90	-0.96 ± 1.64	-1.41 ± 1.35

No.	Compound name	LRI (RTX-5ms)*	Compound class	Mean ± SE fold changes ln(X+1) 24 h	Mean ± SE fold changes ln(X+1) 48 h	Mean ± SE fold changes ln(X+1) 72 h
21	1-tetradecanol	1675	alcohol	**1.91 ± 0.99**	0.31 ± 1.73	2.02 ± 1.46
22	heptanal	901	aldehyde	3.77 ± 2.42	-0.93 ± 1.01	1.00 ± 1.71
23	hexadecanal	1818.5	aldehyde	0.01 ± 0.13	-1.19 ± 2.68	-1.85 ± 1.02
24	tetradecanal	1611.2	aldehyde	1.73 ± 0.47	-1.41 ± 0.67	-0.60 ± 1.70
25	2-butanone	576	ketone	2.23 ± 1.15	-0.22 ± 1.81	-0.10 ± 1.09
26	2-nonanone	1091.8	ketone	2.27 ± 0.52	0.37 ± 0.63	0.83 ± 0.58
27	2-pentanone, 3-methyl	749.1	ketone	**2.54 ± 1.25**	0.06 ± 2.71	-0.18 ± 1.16
28	3-pentanone	700	ketone	2.69 ± 2.62	**4.91 ± 0.89**	5.48 ± 2.09
29	decanoic acid	1369	fatty acid	0.02 ± 1.37	-2.80 ± 1.21	-1.27 ± 1.84
30	gamma-valerolactone-	948.8	lactone	-0.28 ± 0.80	-1.03 ± 0.60	0.14 ± 0.88
31	2-acetyl furan	908.8	furan	0.74 ± 1.43	0.09 ± 1.56	0.99 ± 1.12
32	indane	1031	indane	-1.76 ± 2.05	-1.53 ± 1.30	-2.63 ± 1.06

*linear retention index for rtx-5ms column; ** tentative

Table 4.2: Primers sequences for gene expression analysis

Gene	*A. thaliana* locus	Forward Primer	Reverse Primer
GAPC2	AT1G13440	5'-AGTTGTTGACCTCACGGTTAGAC-3'	5'-TTCCTCCTTGATAGCCTTCTTG-3'
PP2A	AT3G25800	5'-CATGCTCCAAGCTCTTACCTG-3'	5'-AATTTGATGTTTGGAACTCTGTCTT-3'
MYC2	AT1G32640	5'-AGGTTGATGTCGGCGTTG-3'	5'-CGTTAACCACCGACATACTCG-3'
VSP2	AT5G24770	5'-ATCTCGAAGCTGCTGGTTTC-3'	5'-TTTGTGTTCGAACCCGTTG-3'
ERF1	AT3G23240	5'-CGGCGGAGAGAGTTAAAGAG-3'	5'-AACACCCATCCTCGTAGCTG-3'
ETR1	AT1G66340	5'-CACCAAAGGCCACTGCTC-3'	5'-GTGGATTTGTCGGTGTTACCAG-3'
PR1	AT2G14610	5'-CTACGCCGACCGACTAAGAG-3'	5'-CTACTCCCGGCCAAGTTCTC-3'
CYP79B2	AT4G39950	5'-AAGAGGTTGTGCTGCTCCG-3'	5'-TCCAAGTGAAACCTTGAAGAAGTC-3'
CYP79F1	AT1G16410	5'-TTGGAACATTGATGGTCAAGAG-3'	5'-TCTCGTCAATGATCGGATTG-3'
CYP83A1	AT4G13770	5'-CTCCTTATCCCTCGTGCTTG-3'	5'-TGTCGTAACCAGCGATCTTG-3'
TPS10	AT2G24210	5'-AACTCTTTACTGCCGCCTTTG-3'	5'-ACTCGGGGAGTTCATCGAGAC-3'
TPS21	AT5G23960	5'-GAGCACATTGTCTCTTTGCTCA-3'	5'-AATCTCCACCAGTCCACCAC-3'
HPL1	AT4G15440	5'-TGGTGATGAGAGACGCTAACA-3'	5'-CCGATCCGGTTTAAATTCCT-3'
ChlADR	AT1G54870	5'-CCTGGCTTGTAACCATTGCT-3'	5'-CACCTCCGTTAGGGTGAAGA
CHAT	AT3G03480	5'-TGTACGGTGGAACCGCTAAG-3'	5'-GGCACGTAGAAGCTCACTCCT-3'
TMT1	AT2G43920	5'-CGCCACTCGTAAGGGTAAAG-3'	5'-TGGATCAGTTGATCTTCTTCCA-3'

Plants were grown for three weeks, after which leaves were subjected to either damage by one fourth instar *S. exigua* larva or 500 µL of 2.4 µmoles JA (pH 3.7) (±– JA, Sigma, St Louis, IL, USA) (van Dam *et al.* 2004) on the fourth leaf from the apex of the plant. JA was prepared in 0.1% Triton X-100 to facilitate application and absorption to the leaf surface (Bodnaryk 1994, Ludwig-Müller *et al.* 1997). Control plants received empty clip cages. In addition, acidic water treatment in 0.1% Triton X-100 (pH 3.7) was used as a control treatment for JA application to control for acid induced responses (van Dam *et al.* 2010). For each treatment, both local and systemic leaves were harvested as separate sample sets 6, 20, 24, 48 and 72 h after induction. Leaves from three plants were pooled together to obtain one sample and three replicate pools were collected for each time point (except acidic water, which had two replicates for each time point) and kept at -80°C till further analysis.

For primer design, orthologous sequences of the respective *Arabidopsis thaliana* gene were collected from all *Brassica* spp. sequences available in GenBank. Primers were designed on conserved stretches within the *Brassica* orthologous sequences, whereby cross-reactivity with paralogous *Brassica* sequences was avoided (Table 4.2). For each sample, 0.5 µg of total RNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., California, USA) according to the manufacturer's instructions. To estimate the amount of contaminating genomic DNA in each sample, negative control cDNA reactions were made by omitting the reverse transcriptase. Subsequently, all samples were diluted twenty-fold with water. For each cDNA sample, qPCR amplification reactions were performed in triplicate.

The qPCR amplification mix was: 5 µl diluted 1st strand cDNA, 0.75 µl forward primer (10 µM), 0.75 µl reverse primer (10 µM), 12.5 µl iQ™ SYBR Green Supermix (Bio-Rad Laboratories Inc., CA, USA), 6 µl H_2O. The qPCR was performed on the MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad

Laboratories Inc., CA, USA) according to the following protocol: an initial denaturation for 5 min at 95°C, followed by 45 cycles of 15 s at 95°C, 15 s at 58°C, 15 s at 72°C. Thereafter, a melting curve analysis was performed to verify that only a single gene transcript had been amplified. To verify that the primers were indeed targeting the right gene, amplification fragments were cloned and sequenced. Negative control cDNA samples that showed amplification within 45 cycles were discarded from the analysis.

Out of several potential reference genes, the two most stable genes, *GAPC2* and *PP2A*, were selected with the geNorm software (http://medgen.ugent.be/genorm/). For quantification of the gene expression, the relative expression levels of the target genes were calculated by normalization with the expression of the two reference genes (Vandesompele *et al.* 2002). Primer pair amplification efficiencies were determined with the LinReg PCR software (Ruijter *et al.* 2009). Thereafter, fold changes in gene expression levels were calculated by dividing the mean normalized expression of treatment group by the mean normalized expression of the control treatment of empty clip cages for the herbivore induction experiment, and acidic water for JA treatment.

Insect preference

In order to observe herbivore and parasitoid preference between 24, 48 and 72 h damaged plants and undamaged plants, an X-shaped olfactometer set-up was constructed that included a cylindrical releasing chamber of 20 cm ø and 9 cm height. The floor of the chamber had an opening of 1.5 cm ø in its centre for introducing the insects. The opening was closed with a rubber stopper. Four detachable cylindrical arms, each of 17 cm length and 5 cm ø wide were fixed to the chamber at 90 degrees angles. The other end of these arms was closed using a net (Figure 4.1).

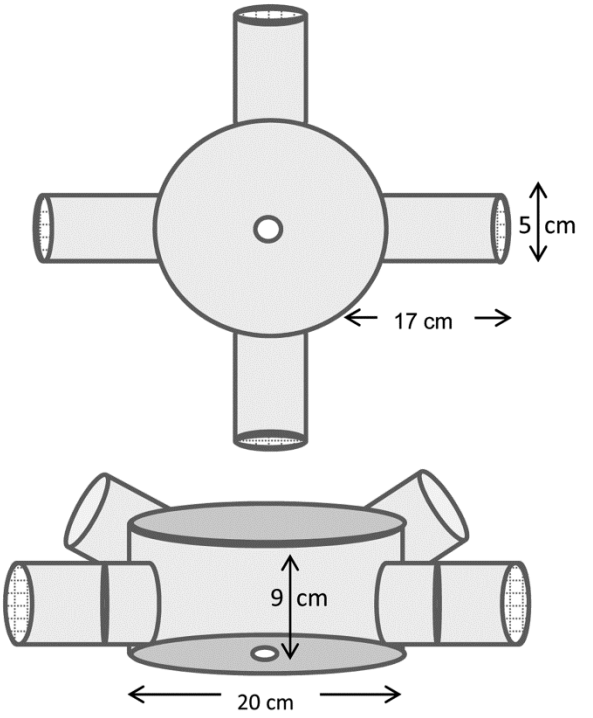


Figure 4.1: Olfactometer set-up for the parasitoid preference test.

The experiment with insect herbivores was conducted in India in November 2009 at Sri Venkateswara College, University of Delhi, Delhi. Plants were damaged for 24, 48 or 72 h prior to the experiment using *S. litura* larva. The adults of both *S. litura* and *P. xylostella* are nocturnal, and therefore herbivore orientation preference experiment was conducted at night when these insects are the most active. The parasitoid orientation experiment was performed in July-August 2009 in the Netherlands Institute of Ecology (NIOO-KNAW), Heteren, The Netherlands. Plants were damaged using *S. exigua* larva in a similar fashion as with the herbivore orientation experiment. The parasitoid orientation experiments were performed in full daylight when both *C. marginiventris* and *C. plutellae* are expected to be the most active.

The damaging herbivores from all three treatments viz., 24 h, 48 h and 72 h, were removed from the leaves prior to the beginning of the experiment. For the experiment, whole potted undamaged plants and plants damaged for 24, 48 and 72 h were placed on either arm of the olfactometer, which was mounted on a tripod stand. In this way, the volatiles of the whole plant could diffuse through the arm closest to the plant. The entire experimental set-up with the olfactometer and all four plants was placed outside at temperatures ranging from 23-26° C in a tent (100*70*70 cm) covered with fine mesh gauze on the roof and all four sides.

Cohorts of females were released in groups of five from the bottom of the chamber and observed for 15 min. When a female moved up to the end of an arm, it was recorded as making a choice for the corresponding plant. After testing five females, the plants were moved to another position to avoid any positional bias in the set-up and the arms of the olfactometer were cleaned with ethanol. After four such replicates of five females each, the test plants were replaced by new ones of the corresponding treatments.

Statistical analysis

For analysing the volatiles, we constructed a separate model for each day of volatile sampling and compared the profiles of the damaged plants collected on that day to the control plants of the same day. The Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) method was applied to this model (Bylesjo *et al.* 2006): the resulting weight vector values reflect the volatile blend emitted from damaged plants compared to that of untreated control plants on each day. The *p*-values were determined from a permutation test where the classification potential of models of identical complexity, fitted based on randomly permuted class labels, such that no response can be expected.

The data of individual volatiles and their gene expression were statistically analysed by the independent sample t-test assuming unequal variances following

the procedures in Rieu and Powers (2009) using SPSS 17.0 (SPSS, Chicago, IL, USA). The calculations for the fold changes and statistical analyses were performed in a similar fashion for volatiles and gene expression values which facilitates comparison of the results.

Insect preference was analysed using non- parametric replicated goodness of fit test with the null hypothesis of no preference (Sokal and Rohlf 1995). Females that did not make a choice were excluded from the analysis.

Results

Volatile analysis

The volatile blends consisted of compounds of various classes including a glucosinolate metabolite, green leaf volatiles (GLVs), a salicylic acid ester, sulfides and terpenes along with other (unidentified) acids, ketones and aldehydes (Table 4.1). For each of the three time points, fold changes in the amount of individual compounds after *S. exigua* feeding compared to control were calculated, and the complete blends were fitted in an OPLS-DA model, comparing the blends over the three sampling days. The overall volatile blends of damaged plants were significantly different between the days (Permutation test of the weight vector value; $p<0.01$). The three discriminant axes represent the contrast between the fold changes of plants damaged for 24 h, 48 h and 72 h (Figure 4.2 a, b and c). The importance of each VOC in each contrast is represented by its position according to its weight vector value on each axis. Increased levels of a compound after 24 h, 48 h or 72 h damage are indicated by a positive weight vector value and reduced levels are shown by a negative value on the respective axis.

The only glucosinolate metabolite emitted, 1-butene-4-isothiocyanate (compound number 1 in Table 4.1), was strongly induced at 24 h after damage, and remained at higher levels thereafter throughout the study period (Independent sample t-test, $p<0.01$ at 24 h and $p<0.005$ at 72 h; Figure 4.2 a, b, c).

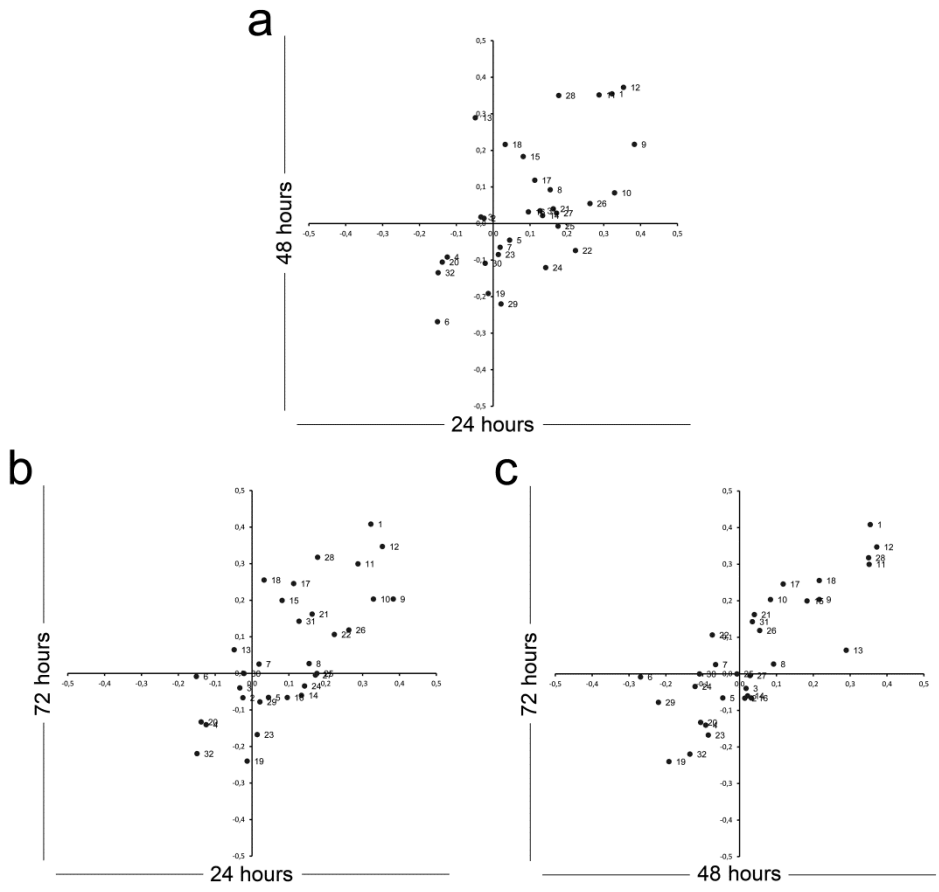


Figure 4.2: Two-dimensional OPLS-Discriminant plots for VOCs emitted by *Brassica juncea* plants due to damage by *Spodoptera exigua* larvae, represented as discriminant functions of damage for 24 h, 48 h and 72 h plotted against each other. The two-dimensional plots show the contribution of each VOC for each treatent group on the X or the Y axis: (a) 24 h vs 48 h, (b) 24 h vs 72 h, and (c) 48 h vs 72 h of damage. Numbers correspond to compounds listed in Table 4.1. The position of each point is determined its weight vector valuein the VOC blend of each treatment group plotted.

Compounds belonging to the GLVs were also emitted at high amounts following herbivory. The emissions of 3-hexen-1-ol (compound number 11) (p<0.05 at 24 h and 48 h) and 3-hexen-1-ol-acetate (compound number 12; p<0.05 at 24 h and 72 h) were significantly increased (Figure 4.2 a, b, c). We also observed a short burst in the emission of acetaldehyde (compound number 13) at 48 h (p<0.05; Figure 4.2 b, c). In addition, acetic acid hexyl ester (compound number 15) emissions showed a marginal increase at 72 h of damage (p=0.058; Figure 4.2 b, c). On the other hand, the levels of acetic acid ethyl ester (compound number 14) and hexadecanoic acid methyl ester (compound number 16) showed no significant change in emission following herbivory (Table 4.1).

Among the terpenes, that constituted the largest number of known compounds in the *B. juncea* volatile blend, we essentially did not find any major changes in monoterpene or sesquiterpene emissions between damaged and undamaged plants (Table 4.1). The only exception was beta-ocimene (compound number 6) that decreased substantially at 48 h, but this change was found to be just not significant (p=0.097; Figure 4.2 a, c). Homoterpenes were the most prominently induced among the terpenes, because both detected homoterpenes, viz. (E,E)-4,8,12-trimethyl-1,3,7,11 tridecatetraene (TMTT) (compound number 9) and <3E>-4,8-dimethyl-1,3,7-nonatriene (DMNT) (compound number 10), were newly synthesized and emitted at higher rates within 24 hours of damage, but inconsistently so, throughout the study period (Figure 2a, b, c). The emission of DMNT was found to be significantly elevated at 72 h of damage (p<0.05), but TMTT did not show significantly increased emissions at any time point tested due to the large variation between replicates (Table 4.1). Both the sulfides detected, viz., dimethyldisulfide (DMDS) and dimethyltrisulfide (DMTS) (compound number 17 and 18, respectively) increased significantly over control levels at 72 h of damage (p<0.05; Table 4.1, Figure 4.2 b, c). The emission of salicylic acid hexyl ester (compound number 19) was decreased after 72 h of *S. exigua* damage, however this induction was not statistically significant (p=0.12; Figure 4.2 b, c).

In addition, several other compounds were affected by *S. exigua* feeding at different time points of the study. Among these, the most prominent compounds were 1-tetradecanol (compound number 21; $p<0.05$) and 2-pentanone-3-methyl (compound number 27, ketone; $p<0.05$), that increased at 24 h of damage, as well as 3-pentanone (compound number 28, ketone; $p<0.01$) that increased at 48 h of damage (Figure 4.2 a, b, c). Other than the volatiles mentioned above, the OPLS-DA model shows several compounds belonging to various classes, such as alcohols, aldehydes and ketones that were prominently different at various time points (e.g. compound numbers 26, 31, 32). However, even though fold changes in case of some compounds were high, we did not find significant changes in their emissions (e.g. compound number 9 at 24 h and compound number 11 at 72 h in Table 4.1). This is due to large standard error values for volatiles from control plants, which is taken into account for the statistical analysis (Rieu and Powers 2009) but not while calculating the fold changes presented in Table 4.1.

Gene expression analysis

The changes in the expression levels of all the tested genes were essentially stronger in damaged (local) leaves than in systemic leaves. In the local leaves, we observed a significant induction of *MYC2* starting at 20 h after caterpillar feeding, which remained at higher levels throughout the study period (Independent sample t-test, $p<0.05$, Figure 4.3 a). *VSP2* was repressed at 6 h, and then was induced at the time points 20 h and 24 h, after which it decreased at 48 h before attaining basal levels at 72 h (Figure 4.3 b).

In the systemic leaves, no response was found for *MYC2*, while *VSP2* was significantly induced two-fold at time points 6 h and 20 h, but was also significantly repressed at 48 h (Independent sample t-test, $p<0.05$). After JA application, we found an immediate strong induction of *MYC2* in local as well as systemic leaves (Independent sample t-test, $p<0.05$). *VSP2* was induced starting from 20 h after JA application in the local leaves, and remained at higher levels throughout the study

period. In the systemic leaves, this gene was induced as early as at 6 h after JA application, reached a peak at 20 h, after which it decreased consistently at the tested time points before reaching basal levels at 72 h. *PR1*, *ETR1* and *ERF1*, on the other hand, principally did not show significant changes in their expression levels in either local or systemic leaves (Figure 4.3 c, d, e).

The expression of genes involved in mono- (*TPS10*) and sesquiterpene (*TPS21*) biosynthesis showed a large biological variation (Figure 4.4 a, b). Following herbivory, both *TPS10* and *TPS21* were slightly induced in the local leaves at 72 h, however this induction was not statistically significant (Independent sample t-test, $p<0.1$). In the systemic leaves, these genes were also slightly induced at 24 h. JA application did not result in any significant changes in the expression levels of these genes. The temporal changes in the genes involved in GLV synthesis were more diverse in their expression patterns, as was also seen in the volatile analysis. *HPL1* was significantly induced in the local leaves at time points 20, 24 and 48 h, while in systemic leaves, this induction was delayed until 72 h after damage (Independent sample t-test, $p<0.05$, Figure 4.4 c). An increase in the *HPL1* expression was observed throughout the study period following JA application, in local as well as systemic leaves. In contrast, *ChlADR* showed a slight decrease at 6 h and a significant decrease at after 48 h of damage in the local leaves. In the systemic leaves, there was a slight increase at 24 h, followed by a decrease after 48 h of damage. JA application decreased *ChlADR* expression at 72 h in the local leaves (Figure 4.4 d). *CHAT* expression showed a trend for increase following herbivory in the local leaves. However, no significant induction or repression was observed in either local or systemic leaves at any of the analysed time points. In contrast, JA application resulted in a slight induction of this gene in local leaves from 20 h to 48 h, in between a significant repression at 6 h and 72 h (Independent sample t-test, $p<0.05$). In the systemic leaves, there was only a slight induction between 6 h and 24 h (Figure 4.4 e).

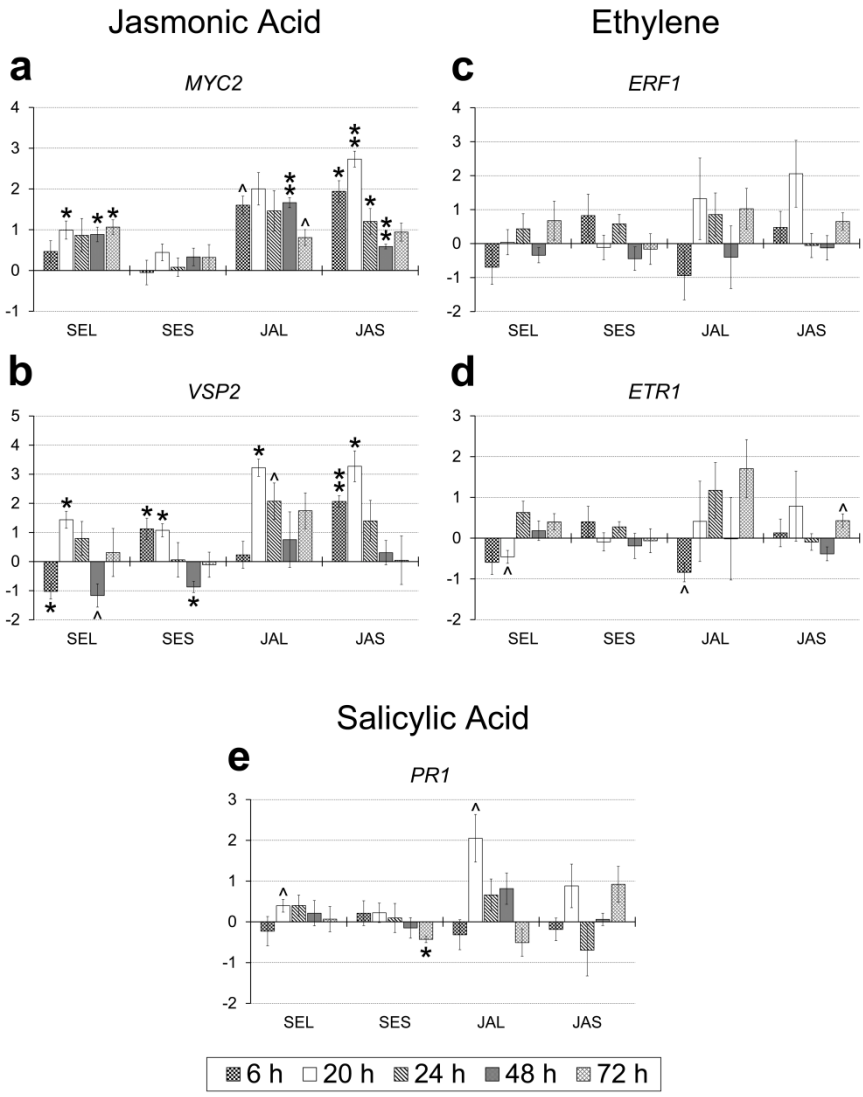


Figure 4.3: Fold changes in mean normalized expression at different time points of treatment versus control for marker genes of the JA, SA and ET hormonal pathways determined using RT-qPCR. SES, systemic leaf after *Spodoptera exigua* induction; SEL, local leaf after *S. exigua* induction; JAS, systemic leaf after JA induction; JAL, local leaf after JA induction. Error bars represent the standard errors; statistically significant fold changes between control and treatment are marked by: ^, $p<0.1$; *, $p<0.05$; **, $p<0.005$ (t-test assuming unequal variances).

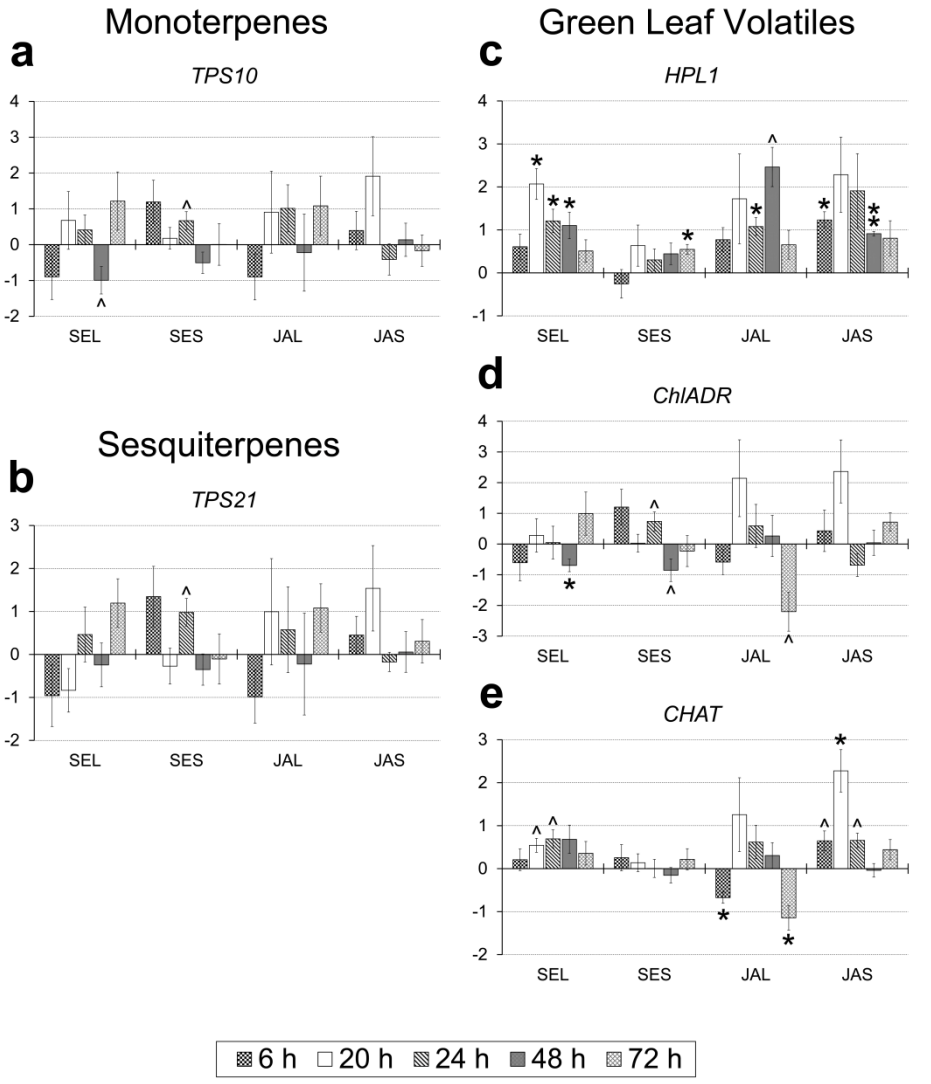


Figure 4.4: Fold changes in mean normalized expression at different time points of treatment versus control for marker genes of the volatile biosynthetic pathways determined using RT-qPCR. SES, systemic leaf after *Spodoptera exigua* induction; SEL, local leaf after *S. exigua* induction; JAS, systemic leaf after JA induction; JAL, local leaf after JA induction. Error bars represent the standard errors; statistically significant fold changes between control and treatment are marked by: ^, $p<0.1$; *, $p<0.05$; **, $p<0.005$ (t-test assuming unequal variances).

Among the glucosinolate genes tested, *CYP79F1* and *CYP83A1* showed a comparable induction pattern in response to herbivory (Figure 4.5 a, b). Both the genes showed induction at 72 h of insect damage in the local leaves (Independent sample t-test; $p<0.05$ for *CYP79F1*; $p<0.1$ for *CYP83A1*). In systemic leaves, *CYP79A1* was repressed at 20 h ($p<0.1$) and increased at 24 h ($p<0.05$). In response to JA application, the expression levels of both genes increased at 6 h in the local leaves ($p<0.1$ for *CYP79F1*; $p<0.05$ for *CYP83A1*). Additionally, *CYP79F1* also showed an increase in its expression levels at 48 h in the local leaves ($p<0.05$), as well as at 6 h ($p<0.05$), 24 h ($p<0.1$) and 72 h ($p<0.05$) of damage in the systemic leaves. In contrast, no significant response due to herbivory was observed for *CYP79B2* in these leaves (Figure 4.5 c). However, after JA application, we observed a significant repression of this gene in the local leaves at 72 h (Independent sample t-test, $p<0.05$). The expression of a gene involved in the synthesis of sulfides (*TMT1*) showed a marked induction in the local leaves when damaged for 20 h, 24 h and 72 h (Independent sample t-test, $p<0.05$). The gene showed a similar trend during the study period in the systemic leaves. *TMT1* also showed a significant increase in its expression levels at all the measured time points except 72 h in the damaged leaf and at 6 h and 20 h in the systemic leaves following JA treatment (Figure 4.5 d).

Insect preference

When offered a choice between undamaged plants and plants damaged for 24, 48, or 72 h, the generalist herbivore *S. litura* significantly preferred undamaged plants (Figure 4.6 a, Replicated G-test, $p<0.01$). Its parasitoid *C. marginiventris*, however, favoured plants damaged for 48 h (Figure 4.6 c, Replicated G-test, $p<0.001$). In contrast, the specialist herbivore *P. xylostella* (Figure 4.6 b; Replicated G-test, $p<0.005$) and its parasitoid *C. plutellae* (Figure 4.6 d; Replicated G-test, $p<0.05$), both preferred plants damaged for 72 h as compared to undamaged plants or plants damaged for 24 h and 48 h.

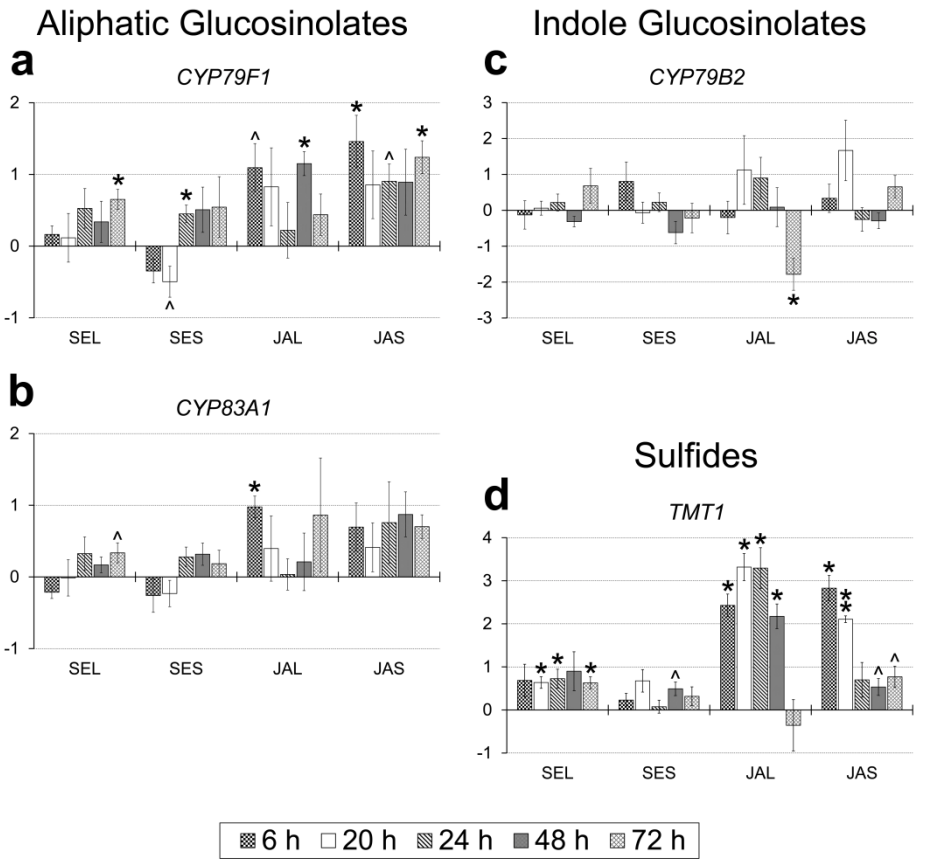


Figure 4.5: Fold changes in mean normalized expression at different time points of treatment versus control for marker genes of the sulfur-containing volatile biosynthetic pathways determined using RT-qPCR. SES, systemic leaf after *Spodoptera exigua* induction; SEL, local leaf after *S. exigua* induction; JAS, systemic leaf after JA induction; JAL, local leaf after JA induction. Error bars represent the standard errors; statistically significant fold changes between control and treatment are marked by: ^, $p<0.1$; *, $p<0.05$; **, $p<0.005$ (t-test assuming unequal variances).

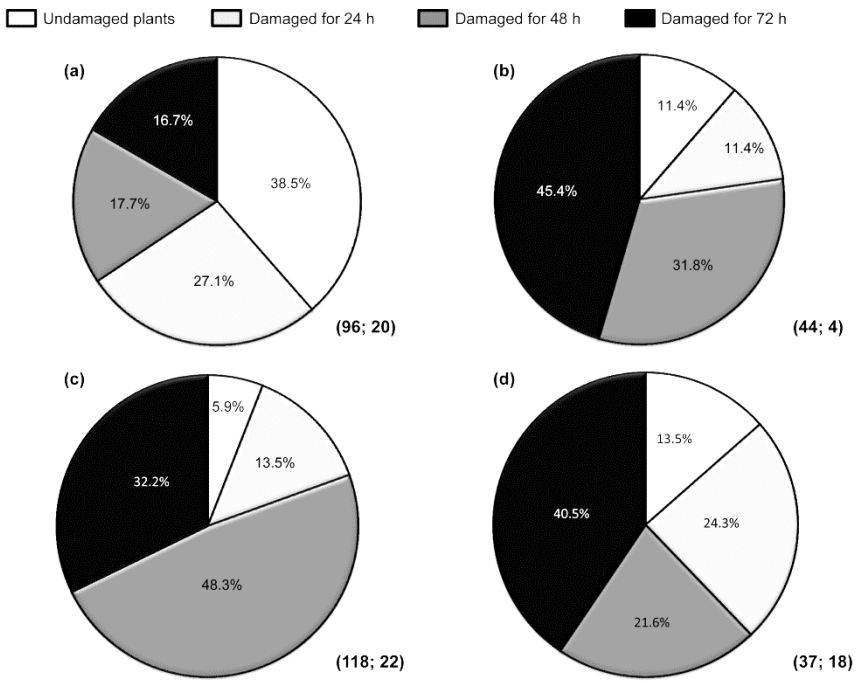


Figure 4.6: Orientation response of naïve adult females of (a) *Spodoptera litura* (n=96) (b) *Plutella xylostella* (n=44) (c) *Cotesia marginiventris* (n=118) (d) *C. plutellae* (n=37) when undamaged plants and plants damaged for 24, 48 or 72 h were offered as a choice. The data were analysed using replicated G test goodness-of-fit and were found significant between the treatments for (a) (p<0.01); (b) (p<0.005); (c) (p<0.001) and (d) (p<0.05). The pie charts represent only the percentage of females that made a choice. Numbers in the parantheses represent the number of females that made a choice and females that did not make a choice and were excluded during the experiment.

Discussion

In this study, we associate damage-induced volatile induction to changes in their gene expression and the effect on herbivores and their parasitoids. We analysed the volatile blends after 24 h, 48 h and 72 h of damage by *S. exigua* larvae and compared them to the volatiles of undamaged plants. Following herbivory, a clear difference was found in the composition of the volatile blends between damaged and undamaged plants as well as between damaged plants measured at all three time points following herbivory. OPLS-DA revealed that a few specific compounds characterized the odour blend of plants damaged at each time point.

The prominent volatiles in the HIPV blends of *B. juncea* were 1-butene-4-isothiocyanate, some GLVs and ketones that increased, as well as homoterpenes TMTT and DMNT that were newly synthesized and also emitted at higher levels following herbivory. Additionally, there was a conspicuous increase in sulfides at 72 h and decrease in the monoterpene beta-ocimene, salicylic acid hexyl ester and decanoic acid at 48 h. Interestingly, no volatiles were found to decrease at 24 h after damage. The gene expression analysis of the signalling pathways and various classes of volatiles mirrored the temporal patterns of volatile induction. Moreover, *S. exigua* was found to induce the JA pathway, while the SA pathway was downregulated. Adult preference bioassays revealed that the generalist *S. litura* was attracted to undamaged plants, whereas the specialist *P. xylostella* preferred plants damaged for 72 h. The parasitoids of both of these herbivores were attracted to damaged plants. However *C. marginiventris*, the parasitoid of *S. litura*, preferred plants damaged for 48 h, whereas *C. plutellae*, corresponding to the preference of its host *P. xylostella*, preferred plants damaged for 72 h.

Temporal dynamics in volatile emission are vital for the infested plant, since early emission of HIPVs would attract the natural enemies more quickly (Loughrin *et al.* 1994). When the VOC blend changes immediately after herbivore damage, the volatiles are mainly released from a stored pool (Paré and Tumlinson 1997). However, often there is a response delay between the beginning of herbivore damage and the release of induced VOCs, which could be explained, in part, by the time needed to activate genes responsible for their biosynthesis (Arimura *et al.* 2004). Here we show that the temporal dynamics of different compound classes of volatiles that are emitted after herbivore damage are also reflected in their gene expression patterns.

Sulfur containing volatiles are characteristic of volatile blends emitted by Brassicaceae and are known to attract specialist herbivores to *Brassica* plants (Mewis *et al.* 2002, Renwick *et al.* 2006, Gols *et al.* 2011). Moreover, the natural enemies of these herbivores respond to damage-induced variation in the concentration of sulfides and glucosinolate breakdown products as cue for the

presence of their hosts (Mumm *et al.* 2008a, Pierre *et al.* 2011). Following herbivory, we found that 1-butene-4-isothiocyanate, the only volatile glucosinolate product detected in our studies in *B. juncea*, increased consistently after damage, and this increase was also reflected in the gene expression activities of alkenyl glucosinolate biosynthesis genes (Figure 4.5). Earlier studies revealed that increased levels of isothiocyanates are highly attractive to *P. xylostella* (Pivnick *et al.* 1994). In our study, 1-butene-4-isothiocyanate emission was found to be the highest at 72 h, and correspondingly in the herbivore preference bioassay, the female *P. xylostella* also preferred these plants. This suggests that 1-butene-4-isothiocyanate plays an important role in the attraction of this specialist herbivore to *B. juncea* as well. The emissions of other sulfur containing compounds, viz. DMDS and DMTS, were also increased after 72 h of damage. Accordingly, *TMT1*, a thiol methyltransferase that methylates the hydrolysis products of glucosinolates and converts them into volatile sulfur compounds such as DMDS and DMTS (Attieh *et al.* 2000), was also positively induced in our gene expression analysis. Earlier studies by Reddy *et al.* (2002) have established that *C. plutellae*, the parasitoid of *P. xylostella*, was attracted to these chemicals. In our study, these parasitoids were attracted to plants damaged for 72 h which also emitted the highest levels of sulfides, underscoring the possible role of these HIPVs in attracting these natural enemies to damaged plants. Remarkably, the plants not only emitted DMDS and DMTS at this time point, but also attracted *P. xylostella* adults. Therefore, *P. xylostella* female attracted to this plant for oviposition may enhance the chances of their offspring being parasitized. However, our results are too preliminary to say whether these females are an example that "mother does not always know the best" or whether long term fitness gains may play a role in this seemingly counterintuitive result (Mayhew 2001).

Terpenoids and GLVs often comprise a large and diverse portion of the volatile blends emitted by intact as well as damaged Brassicaceous plants (Van Poecke *et al.* 2001, Mumm *et al.* 2008b). In herbivore-damaged *B. juncea*, most of

the GLV compounds increased after damage, and the expression profile of the two of the three genes associated with their production showed a similar pattern. In contrast, the emission of beta-ocimene, a monoterpene, had decreased within 48 h of initial damage. In *Arabidopsis thaliana*, TPS10 is involved in the synthesis of this compound (Bohlmann *et al.* 2000) and the activity of this gene was indeed slightly repressed both in local and systemic leaves in damaged plants. Sesquiterpenes did not show any reaction to damage either in the volatile analysis or at the level of gene expression. On the other hand, the two homoterpenes, *viz.,* TMTT and DMNT, were produced *de novo* following herbivory. In other plants species, these compounds were also found to be newly produced or increase in quantity following damage or treatment with elicitors (Turlings *et al*. 1990, Van Poecke *et al.* 2001, Herde *et al.* 2008, Pierre *et al.* 2011).

Both GLVs (Mattiacci *et al.* 1994, Halitschke *et al*. 2008) and terpenes (Degenhardt *et al.* 2003, De Boer *et al.* 2004) are well known to attract natural enemies of herbivores. McCormick *et al.* (2012) described three possible means by which these carnivores discriminate odour: (1) species-specific odour recognition, in which the carnivore separates volatile compounds restricted to a single species or group of related species of herbivores; (2) ratio-specific odour recognition, whereby a ratio of compounds in the blend of volatiles is recognized; (3) whole-blend odour recognition, in which the entire blend or many of its components are perceived as a whole. The ability of a parasitoid to distinguish between volatile blends depends on the dietary specialization of both the parasitoid and its herbivore host (Vet and Dicke 1992, Steidle and van Loon 2003). Specialist parasitoids cannot rely solely on induced terpenoids and GLVs for their host identification, because these compounds are not specific to any particular herbivore damage and hence do not give a reliable cue of the presence of their host (van Dam *et al.* 2010, Gols *et al.* 2011). On the other hand, generalist parasitoids that attack several herbivores feeding on plants of different families may rely on more generalized cues (Gols *et al.* 2012). Earlier studies have demonstrated that the generalist parasitoid *C. marginiventris* takes cues

from terpenoids and GLVs for its host location (Turlings *et al.* 1991). Based on our results, we speculate that *C. marginiventris* uses one or more of changes in these compounds after 48 h of damage as cues to locate its host, as the females were most attracted to these plants. Further studies in this direction, such as preference bioassays using individual volatiles and electro-antennogram (EAG) recordings of *C. marginiventris* are needed to determine the mechanisms behind the attraction of these parasitoids to specific compounds. Additionally, we also found that the generalist herbivore *S. litura* strongly preferred to move towards undamaged plants as compared to any of the damaged plants. These studies are consistent with earlier studies that demonstrate that the same HIPVs that attract specialist herbivores and parasitoids can repel generalist herbivores (Dicke and Vet 1999, De Moraes *et al*. 2001, Karban and Baxter 2001).

Our studies are in accordance with earlier studies that have shown that many parasitoids and specialist herbivores associate a specific blend odour with the presence of hosts (Turlings *et al.* 1993, Vet *et al.* 1995, Bruce *et al.* 2005). To date, more than 25 species of natural enemies in the third trophic level are known to be attracted to HIPVs (Mumm and Dicke 2010, Reddy 2012). However, the chemical diversity of HIPVs makes it difficult to establish which of the blend components may evoke a response in naïve parasitoids. In fact, earlier studies aimed at identifying the minimal blend showed that some compounds in the complete blend may mask the attractive components (Turlings and Fritzsche 1999). Therefore, the value of attraction is believed to be determined by the relative reliability and detectability of plant volatile signals (Vet *et al.* 1991). By showing different blends of volatiles at different time points, our study provides a fair explanation for the differential preference of the parasitoids to these plants. Since our study combines the dynamics of HIPV blend with the behaviour of insect herbivores and their parasitoids, we can infer the ecological importance of these dynamics. However, as the most important odour cue for the generalist parasitoids may be determined by specific ratios of the emitted volatiles within the blend, it is challenging to pinpoint which compounds are the most important (Gols and Harvey 2009).

In addition, many studies showed that parasitoids can enhance the specificity of their responses to HIPVs through associate learning, which is not included in our experimental set-up as we used naïve females for the preference tests (Vet *et al.* 1995, Geervliet *et al.* 1998, Vet 1999). Moreover, we performed the orientation studies for parasitoids during the day because they are most active at that time, and for the herbivores at night because both *S. litura* and *P. xylostella* are nocturnal insects. However, we analysed the volatile blends only during the day and therefore they are not completely representative for the preference experiments of the nocturnal adult herbivores. Nevertheless, we expect that similar temporal dynamics of volatile emission is the mechanism that explains the adult herbivore preferences [but see De Moraes *et al.* (2001)].

Our gene expression studies of the signalling pathways showed that, similar to the response observed after JA application, *MYC2* and *VSP2* were both significantly induced after *S. exigua* larval feeding, indicating that this herbivore induces the JA pathway in *B. juncea*. We also found similarities in the expression levels of genes involved in glucosinolate and volatile biosynthesis after larval feeding and JA application. In contrast, there were no significant changes in the expressions of genes involved in the SA or ET pathways; *PR1* was even repressed by 72 h of damage. This clearly indicates that the responses induced by *S. exigua* feeding are mainly controlled by the JA pathway in *B. juncea*. Studies indicate that, although the octadecanoid pathway is the key pathway in the induction of volatile production, many herbivore-induced induction responses are co-regulated by the ET and SA pathway (Dicke *et al.* 1999, Genoud and Metraux 1999). A study on *N. attenuata* showed that components in *S. exigua* oral secretions can suppress the JA pathway by activating the SA pathway, thus indicating that SA functions antagonistically with the octadecanoid pathway (Diezel *et al.* 2009). This antagonism may be dose and timing-dependent as is described in different *Arabidopsis* accessions (Koornneef and Pieterse 2008). The JA and ET pathway converge in the transcriptional activation of *ERF1*; increases of ERF1 expression would indicate a synergistic response of JA and ET pathways

(Lorenzo *et al.* 2003). However, in *B. juncea*, though herbivory by *S. exigua* activates the JA-signalling pathway, we did not find any significant activities in the ET and SA pathways. Our studies thus contradict earlier studies demonstrating that the elicitors from salivary secretions of *S. exigua* induce SA pathway (Weech *et al.* 2008, Diezel *et al*. 2009, Verhage *et al*. 2010).

In the gene expression analysis, the observed quantitative differences in fold changes of the above mentioned genes between the JA application treatment and the larval feeding can probably be explained by the amount of JA involved. While the JA application treatment consisted of a one-time JA induction with 500 µg JA per leaf, the larval feeding resulted probably in a more gradual increase of internal JA levels. We observed that several of the fold changes had a large standard error and therefore were not always statistically significant, which may partly be because of analysing only three biological replicates. Since we were mainly interested in the temporal dynamics of the induced responses, we had to opt for a balance between the number of different time points and biological replicates that we could measure. Moreover, the variation in the gene activities and VOC emissions may partly be explained by the biological variation between the individual plants tested. However, we suspect that the major part of the biological variation was due to the differential amount of feeding by individual larvae and the time at which they initiated feeding, and thus causing damage to the plant.

In summary, our study has demonstrated that HIPVs allow herbivores as well as their parasitoids to discriminate between herbivore infested plants subjected to different time periods of damage. Furthermore, the responses of the insects are dependent on temporal variations in the emissions of volatiles after initial feeding damage to plant tissues. This temporal pattern is also reflected in the dynamics of the genes involved in the synthesis of the volatiles and their precursors. Our study thus provides a comprehensive analysis of the mechanisms underlying temporal patterns of HIPV emissions and their function in natural environments.

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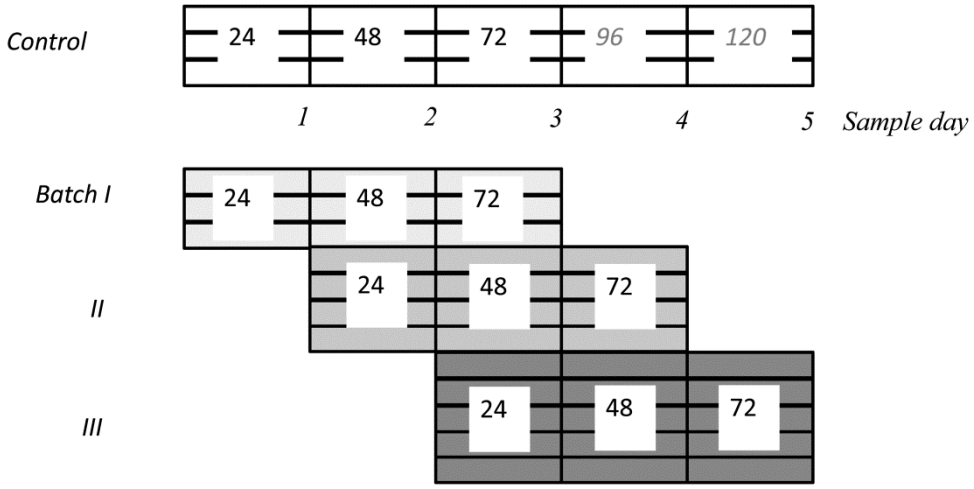

Supplementary Figure 1: Experimental design for volatile collection. The plants were used in three batches: Batch I, II and III had 3, 4 and 5 damaged plants, respectively (indicated by small rectangles in boxes). The same three undamaged plants were used in the whole experimental period. The numbers inside the boxes indicate time for which damage has occurred (in hours) and the number outside the boxes represent the day of the sampling.

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Chapter 5

Composition, structure and function of extrafloral nectaries in *Brassica juncea* (Brassicaceae)

Picture by: Sonia Dourlot

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Abstract

While nectaries are commonly found in flowers, some plants also form extrafloral nectaries on stems or leaves. However, extrafloral nectaries have not been reported to occur in the family Brassicaceae. We investigated the structure and function of extrafloral nectaries in *Brassica juncea* (L.) Czern., belonging to this family. The extrafloral nectar (EFN) was secreted from previously amorphic sites on stems, flowering stalks and leaf axils from the onset of flowering until silique formation. Transverse sections of the stem at the point of nectar secretion revealed a pocket-like structure whose opening was surrounded by modified stomatal guard cells. The EFN droplets were viscous and up to 50% of the total weight was sugars, 97% of which was sucrose in the five varieties of *B. juncea* examined. Among amino acids, threonine, glutamine, arginine and glutamate were the most abundant. We also found the glucosinolates, mainly consisting of gluconapin and sinigrin in the EFN droplets. Nectar secretion was increased when the plants were damaged by chewing above- and belowground herbivores, and by sap-sucking aphids. Parasitoids of each herbivore species were tested for preference and survival on the nectar. We found three parasitoids preferring EFN and sucrose solutions over water. Also, their survival and fecundity was positively affected by feeding on EFN. Based on these results, we conclude that EFN production in *B. juncea* can influence the arrestment of nectar-foraging parasitoids and is capable of sustaining them, and thereby might contribute to the indirect defense of this plant species.

Introduction

Extrafloral nectaries are nectar-secreting parts of the plant surface other than flowers. These organs are not directly involved in pollination, and can be found on virtually all aboveground plant parts (Koptur 1992, Oliveira and Pie 1998). Plants bearing extrafloral nectaries are widely distributed around the world. One hundred and fourteen plant families with more than 700 genera and 4,000 species of plants, including pteridophytes, gymnosperms and angiosperms are known to possess extrafloral nectaries to date (Keeler 2008).

Extrafloral nectaries display a wide diversity in structure as well as composition of nectar between and within plant families. They may be found as structural outgrowths on the cuticle, or situated in the mesophyll of the plant. The simplest, and perhaps the more primitive nectaries are 'gestaltless', *i.e.*, without any externally visible structure (Zimmermann 1932, Frey-Wyssling and Häusermann 1960). These nectaries can only be identified as areas where nectar appears on the plant surface. Consequently, these amorphic nectaries are difficult to identify when no nectar is secreted, and may be strongly under-reported. Commonly, the extrafloral nectar (EFN) is released through modified stomata that remain permanently open, or through specialized trichomes (Schmid 1988, de la Barrera and Nobel 2004). Furthermore, many nectaries are characterized by a continuous cuticle present on the surface of the nectar epidermis that may have specialized pores called 'secretory pits' or narrow tubular interruptions called 'microchannels', through which nectar can be exuded. Alternatively, the cuticle might simply rupture as a result of built-up pressure to release nectar (Escalante-Pérez and Heil 2012). This structural and mechanistic diversity of extrafloral nectaries could be due to their recurrent gain or loss in the course of evolution, and different selective pressures on their functionality in different plants (Heil 2011).

Phloem supplies both floral and extrafloral nectaries. Consequently, the chemical composition of EFN is essentially similar to that of phloem and floral

nectar. It is mainly composed of sugars, but may also contain amino acids, and sometimes a blend of other compounds such as inorganic ions, proteins, lipids, organic acids, phenolics, alkaloids and terpenoids. New compounds belonging to a variety of classes are still being discovered in EFN. Its composition differs in quality and quantity between different plant taxa and can be changed by biotic and abiotic stress factors in the environment (Nepi *et al.* 2009, Heil 2011).

The ecological function of EFN has been controversial for quite some time. In the past two decades, many studies have shown that EFN can play an important role in constitutive and induced plant defenses against herbivores (Wackers and Bezemer 2003, Heil *et al.* 2004, Lach *et al.* 2009, Heil 2011, Holland *et al.* 2011). Extrafloral nectaries act as indirect defenses by providing an alternative food source for natural enemies of herbivores, such as parasitoids, predators and mutualistic ants. The presence of freely available carbohydrates and amino acids, along with other nutrients, makes EFN a wholesome nutritional resource for the natural enemies, thus enhancing the prospects of them staying on the plant. Ants may even protect food resources such as EFN, thereby removing herbivores from the plant (Bentley 1976). EFN production may be increased when the plant is damaged by herbivores, and collectively with other direct and indirect defenses, this adds to the defense strategies of the plant (Heil 2008).

Although many families are known to have EFN, it has not been reported in the Brassicaceae (Keeler 2008). Here we describe extrafloral nectaries in Brown/Indian mustard, *Brassica juncea* (L.) Czern., a species belonging to this family. We analyze the distribution and ultrastructure of these nectaries on the plant, and the chemical composition of the secreted EFN. We also experimentally investigate their possible ecological function by studying the induction of EFN production by the above- and belowground insect herbivores of *B. juncea*, belonging to different feeding guilds. Additionally, we test the attraction, survival and fecundity of the parasitoids of these herbivores on EFN to explore the possibility that EFN contributes to the indirect defense strategy of this plant species.

Materials and methods

Experiments were performed when plants were approximately four weeks old and in stage 63 according to BBCH scale (Lancashire, *et al.* 1991). EFN composition in five varieties of *B. juncea* and temporal dynamics of EFN induction when the plants were damaged by *Spodoptera exigua* were examined at NIOO-KNAW, Heteren (pre-Wageningen location), the Netherlands (NL). Studies on the temporal dynamics of EFN induction with insects of different feeding guilds as well as parasitoid preference and survival bioassays were performed in UMR BiO3P, University of Rennes, Rennes, France. Histochemical studies were done at the University Jean Monnet, Saint-Etienne, France.

Plants

Seeds of five commonly grown varieties of *B. juncea*, *viz*., *varuna, P. bold, P. bahar, P. agarni* and *JDG*, were obtained from the Division of Genetics, IARI, New Delhi, India, and stored dry and in the dark at 10°C. They were germinated on glass beads in water in 10x10 cm plastic containers with a clear plastic lid.

In NL, the greenhouse was kept at 21°C during the day and 16°C at night, at 16h/8h LD conditions maintained by sodium lamps. Seven days later, seedlings were transferred to 1.8-L pots, containing 1000 g peat soil-sand mixture (Lentse Potgrond no. 4, Lent, NL). From third week onwards, plants were supplied with 0.5 Hoagland solution (Hoagland and Arnon 1950) once a week. Plants were maintained at 65% RH throughout their growth period, but were transferred to a climate chamber with 90% RH 24 h prior to the experiments.

In Rennes, the plants were grown in a greenhouse at 20+/-2°C; 65% RH 16h/8h LD conditions and supplemented once with 4N:6P:8K and micronutrients fertilizer from the third week onwards. During third week, they were transferred to plant growth room with conditions similar to the greenhouse to acclimatize them prior to the experiments.

For histochemical studies, plants were grown in the greenhouse at Saint-Etienne University, France, at 21+/-2°C; 60% RH; 12h/12h LD conditions and supplied with 2N:6P:7K fertilizer.

Induction and histochemical studies were conducted using the variety *varuna*. Sugar and amino acid composition of EFN was analyzed using *varuna*, *P. bold, P. bahar, P. agarni* and *JDG* and glucosinolate composition was determined using *P. bold, P. bahar, P. agarni* and *JDG.*

Insects

Insect cultures were maintained in a climate room at 27°C, 50–70% RH, and 16h/8h LD photoperiod. *Pieris brassicae*, *Myzus persicae* and *Delia radicum* and its parasitoid, *Trybliographa rapae* were used from the culture maintained in UMR BiO3P, University of Rennes. *D. radicum* and *T. rapae* were reared according to Neveu *et al.* (1996). *M. persicae* was reared on *B. nigra* and *P. brassicae* was reared on *B. oleracea* plants. Cocoons of *Cotesia glomerata* (parasitoid of *P. brassicae*) and *Diaeretiella rapae* (parasitoid of *M. Persicae*) were obtained from NIOO-KNAW, NL and University of Rennes, France, respectively. *Spodoptera exigua* was obtained from Wageningen University, NL, and maintained on artificial diet (Vickerman and Trumble 1999). The cocoons of its parasitoid, *C. marginiventris*, were obtained from University of Neuchâtel, Switzerland. Parasitoid females were used in the experiments within 24 h of emergence.

EFN collection

Experiments were conducted within 24 h of appearance of EFN on plants. As the amount of EFN produced per plant was too small to be weighed accurately, EFN produced by one group of five plants was pooled. For each treatment group (n = 15), plants were divided into three groups of five approximately similarly sized plants, unless mentioned otherwise.

EFN drops were absorbed using two circles punched out of Whatman filter paper no.1 for each treatment group. These papers were weighed on Mettler-Toledo MT5 Electrobalance (accuracy ±1 μg) prior to and after EFN collection. Thereafter, the filter paper was immersed in 1.5 ml MilliQ water and allowed to remain at room temperature for one hour so that nectar would dissolve in water. The Eppendorf tubes were stored at -20°C until further analysis. Total EFN

collected was determined by calculating the difference between the weights before and after the collection from each group. The average EFN per plant was determined by dividing the total of a group by 5.

Histochemical methods

To study the structure of tissue that secretes EFN, paradermal sections were observed beneath droplets directly by photonic microscopy (Leitz DMRB microscope). For transverse sections, the area was marked around the droplet using a marker pen and cut manually with a razor blade. Fresh sections were stained with Toluidine blue O (Parker *et al.* 1982) or with Rawlins-Takashashi reaction (Rawlins and Takahash 1952) to identify the tissues. Stem surface was observed by ESEM after drying the droplets with a tissue. Stem samples were laid on a stage fitted to the low-pressure chamber of an S-3000N Hitachi microscope (Tokyo, Japan). To allow ESEM observations, samples were cooled from 4°C to a minimum of –20°C by the Pelletier effect. Pressure was then set at 110 Pa and tension at 15 kV.

EFN composition

Sugar and amino acid analysis was performed according to van Dam and Oomen (2008). For soluble sugars, analysis was done on Dionex HPLC system, using a Carbopac PA1 column (2 x 250 mm) and a Carbopac PA1 guard column (2 x 50 mm) (Dionex). Separation of sugars was done with isocratic gradient mixture of 10% 1M NaOH and 90% MilliQ water at a flow rate of 0.25 ml per min.

Amino acid concentration was analyzed on a Dionex HPLC system by integrated pulsed amperometric detection. Amino acids were separated with a ternary gradient on a 2 x 50 mm AminoPac© PA10 column with a 2 x 50 mm AminoPac© PA10 Guard column (Dionex). Sigma AAS- 18 amino acid standard (Sigma) containing 18 amino acids was supplemented with asparagine, glutamine and tryptophan (2.5 μmoles ml^{-1} each) to obtain a reference sample containing the 20 most common amino acids.

For glucosinolate analysis, EFN was collected from five plants of approximately similar size and dissolved in 1 ml water in an Eppendorf tube and frozen at -20°C till further analysis. Ten such samples were pooled together to obtain one biological replicate. Three such biological replicates were made per cultivar. Each sample was directly added to a DEAE-Sephadex A-25 column (5 x 10mm). The column was washed twice with 1 ml 70% MeOH, once with 1 ml MilliQ and then twice with 1 ml 20 mM NaOAC buffer (pH 5.5). 20 μL of aryl sulfatase (Sigma type H-1 of Helix pomatia) was added to the columns and flushed down with 50 μL NaOAC buffer. The columns were then covered with aluminium foil and incubated overnight at room temperature. Thereafter, desulfoglucosinolates were eluted from the columns with 0.75 ml MilliQ water twice, and freeze-dried. The residue was redissolved in 1 ml of MilliQ water and stored in -20°C deep freezer until further analysis.

Glucosinolates were analyzed according to van Dam *et al.* (2004) using a photodiode array detector with the integration wavelength of 229 nm (Dionex, Sunnyvale,CA, USA). Desulfoglucosinolate peaks were identified by comparison of retention times and UV spectra with a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R) and authentic standards (progoitrin, gluconapin, glucoiberin, glucobrassicanapin, glucotropeaolin, gluconasturtiin, glucoraphanin, glucoerucin, glucobrassicin, sinalbin; Phytoplan, Heidelberg, Germany).

Results of the analysis were calculated back to the mass of collected nectar in order to calculate the sugar, amino acid and glucosinolate concentrations in EFN.

Time course of induction

Plants were divided into four groups for damaged and control treatments. Each group contained five approximately similarly-sized plants (n = 20). EFN droplets were removed with the help of a filter paper prior to the experiment. A single fourth instar *S. exigua* larva was introduced in a clip cage on the fourth leaf counted from the apex of the plant at noon and readings were taken every 8

hours for the first two days. After that, EFN was collected every 16 hours till EFN production had ceased. Control groups received empty clip cages. Groups of damaged and undamaged plants were arranged randomly. The larvae remained on the plants for the whole experimental period.

Induction by insects of different feeding guilds

EFN droplets were removed using a filter paper prior to the experiment. A single fourth instar *S. exigua* or *P. brassicae* larva was placed in a clip cage on the fourth leaf counted from the apex of the plant. When the larvae had eaten most of the leaf, they were transferred to another leaf of the plant. Five-third instar larvae of *D. radicum* were placed onto the soil surface using a brush adjacent to the roots of each plant. Plants were examined 30 minutes later to ensure that all the larvae had moved into the soil. Ten nymphs of *M. persicae* were introduced to the fourth leaf of the plant but they were not restricted by any means, and were allowed to move freely on the plant. Each day the number of aphids was counted and new aphids were added to compensate for any losses.

Control groups of plants with *S. exigua* and *P. brassicae* treatments received empty clip cages and with *M. persicae* and *D. radicum* treatments were left as they were. The larvae remained on the plants for the whole experimental period. EFN was collected every 24 h for five consecutive days after the insects were introduced.

Parasitoid preference

Prior to the experiment, 0-24 h old naïve females of *C. marginiventris* (n = 51), *C. glomerata* (n = 115), *T. rapae* (n = 98) and *Di. rapae* (n = 64) (where n represents the total number of parasitoids tested including those who did not make a choice) were collected after emergence and separated individually into vials. During the experiment, 5 μL droplets of water, EFN and sucrose solution were placed equidistant from each other in a 6 cm diameter Petridish. The sucrose solution prepared was of the same concentration as that of EFN to keep the sugar content similar (300μg EFN constituting 50% sucrose in 100μL of water or 150μg of

sucrose in 100μL of water). Parasitoids were released individually in the center of the Petridish. The first droplet on which they started feeding was recorded as their choice. Each female was observed for a maximum of 15 minutes or until her first choice, whichever was earlier, after which she was removed from the Petridish. Females that did not respond within this time frame were put in the "no decision" category and were discarded from the experiment.

Parasitoid survival and fecundity

The experiment was conducted in a climate room (19°C, 50-60% RH, 16h/ 8h LD). A total of 15 parasitoids per species were tested per treatment group. Parasitoids were placed individually in a Petridish of 9 cm diameter containing 10 μL of either EFN or sucrose solution for 24 h. The control group received 10 μL of water. The following day, all the parasitoids were provided only with water in moist cotton until the end of the experiment. Survival of the parasitoids was scored daily. For determining fecundity, females of *C. marginiventris* and *T. rapae* were dissected after they died in the survival experiment and the number of eggs in the ovaries was counted using a compound microscope.

Statistical analysis

Data were analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA) unless stated otherwise. Normality and homogeneity of variance for all the data obtained were determined using one-sample Kolmogorov–Smirnov test and Levene's test, respectively.

Based on EFN collected from plants of *B. juncea* var *varuna* (n = 171), there was no correlation found between plant weight and absolute weight of EFN (Pearson Correlation; $P > 0.05$). Therefore, we present the absolute amount per plant throughout this paper. Concentration of sugars and glucosinolates were compared using univariate ANOVA and the concentration of amino acids was compared using non parametric Kruskal- Wallis test. The overall amount of EFN induced and its sugar content in plants damaged by *S. exigua* were analyzed using repeated measure ANOVA and individual time points were analyzed using *t*-tests with sequential Bonferroni corrections (Holm 1979). Induction of EFN due

to damage by herbivores was analyzed using repeated measure ANOVA and the effect of Days and Days x Damage was tested on EFN production. Orientation of parasitoids between water, EFN and sucrose solution was analyzed using a non-parametric Chi- square test.

Survival analysis was conducted by using the R software R Development Core Team (2011), additional R packages 'survival' (Therneau and Lumley 2009) and *RVAide Memoire* (Hervé 2011). Influence of diet on parasitoid survival was assessed by using a Cox proportional-hazards regression model. Each species was fitted into a model. Proportionality of hazards was tested and assumed for all four models. For each species, treatment effect was assessed by a one-way deviance analysis and a chi-square test. When treatment had a significant effect on female survival, pairwise comparisons between EFN, sucrose and water were performed by using the log-rank test. *P*-values were adjusted using Benjamini and Hochberg correction. Parasitoid fecundity data was analyzed using univariate ANOVA with Bonferroni corrections.

Results

Morphology and anatomy

Under sufficient light conditions, EFN droplets were secreted in *B. juncea* at the onset of flowering when plants were at stage 63 according to BBCH scale (Lancashire *et al.* 1991). These nectaries were found on the plant stem, peduncles and in leaf axils (Figure 5.1a and b).

Secretion started at the flowering stage and continued for 5-8 days until silique formation. The average weight of these droplets (n = 230) was 0.28 ± 0.02 mg. Occasionally droplets weighing more than 2 mg were found. The total mass of EFN droplets on plants was the highest on the first day of their appearance, and decreased with each successive removal. EFN secretion was observed in the greenhouse in NL and France and under field conditions in Delhi-India (V. Mathur and N. M. van Dam, personal observations) between October and February, which is the typical growing season of mustard in India.

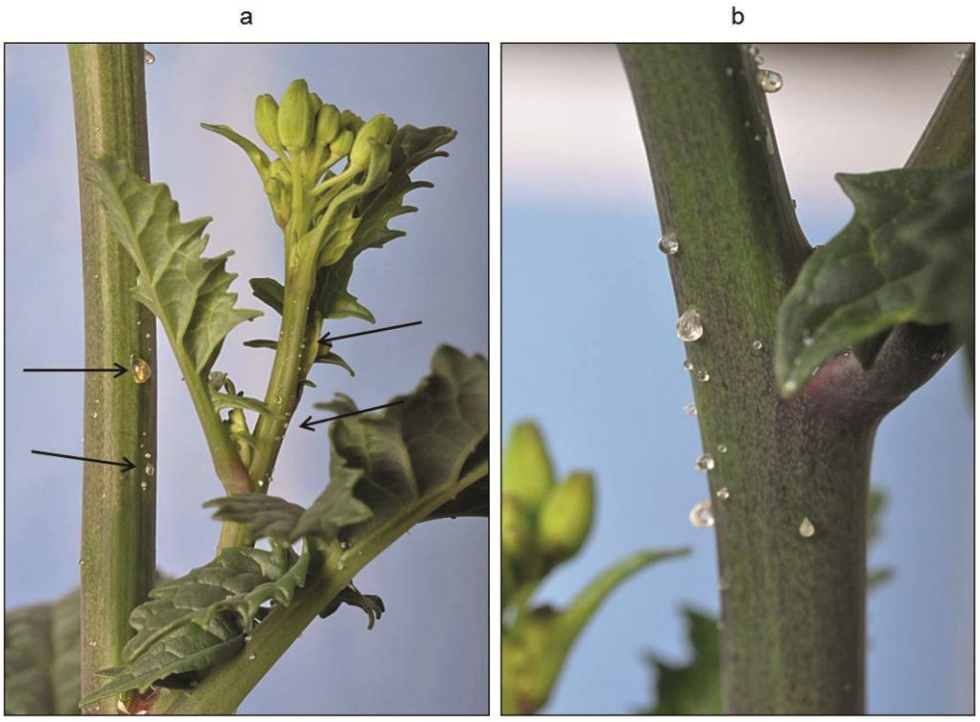

Figure 5.1: Extrafloral nectar (EFN) in *Brassica juncea*. EFN is secreted from the stem, peduncles and in leaf axils of the plant between the flowering stage until silique formation. (© S. Dourlot, University of Rennes 1, UMR IGEPP).

Nectar droplets were gently wiped from the stem and the epidermis underneath was observed under Environmental Scanning Electron Microscope (ESEM). We found a pore that looked like modified stomata, whose guard cells were always opened, in contrast to typical stomata that were closed (Figure 5.2).

Paradermal peelings observed by light microscopy revealed a lack of green parenchyma in this area. A pocket-like structure of around 50 μm diameter was observed in the transverse sections passing through the pore (Figure 5.3a and b). However, Toluidine blue O and Rawlins-Takahashi staining on the stem anatomy did not provide a detailed picture of the connection between this structure and xylem or phloem.

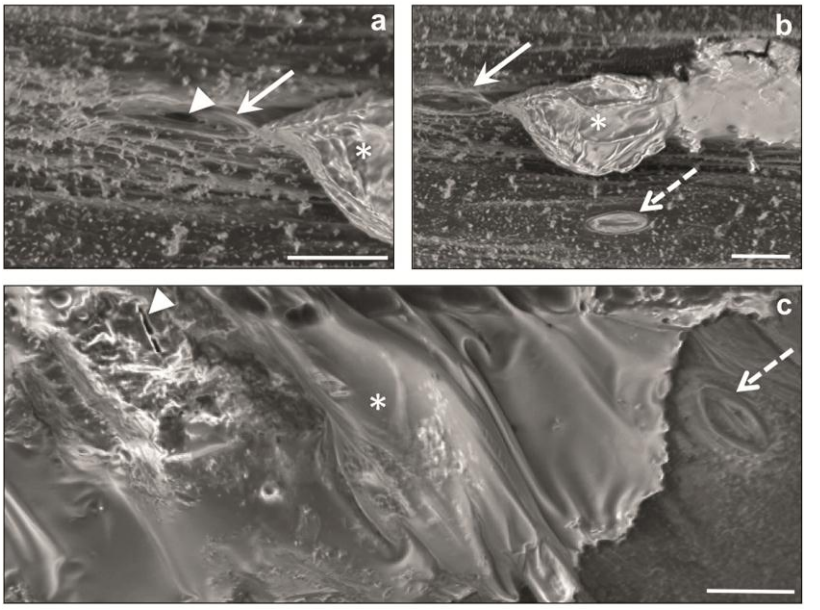

Figure 5.2: Front view of *Brassica juncea* epidermis through ESEM at the site of EFN droplet (a) modified open stomata; (b) wider view of (a) with closed stomata; (c) a modified stomata and a typical closed stomata. Arrows: modified stomata; arrow heads: pore of the modified stomata; dashed arrows: closed stomata. Asterisks indicate a nectar droplet residue. Scale bars 20 µm.

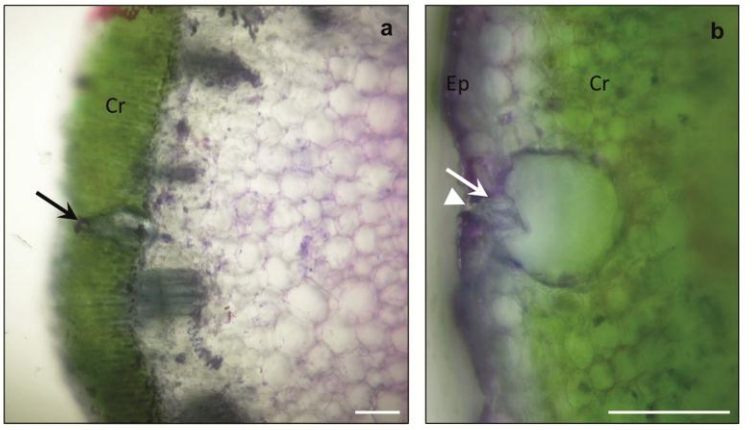

Figure 5.3: Transversal sections of extrafloral nectaries stained with Toluidine Blue O (a) Pocket-like structures are concealed deep within the stem (b) These structures are guarded by an modified stomata (arrow) and secrete nectar by an open pore (arrow head). Ep: epidermis; Cr: cortical parenchyma. Scale bars 100 µm.

EFN composition

Analysis of five commonly grown varieties of *B. juncea*, *viz*., *varuna*, *Pusa bold*, *P. bahar*, *P. agarni* and *JDG*, showed that both EFN sugar and amino acid compositions were comparable in all these varieties. Sugars accounted for almost 50% of the EFN weight in all five varieties and were similar in concentration (Univariate ANOVA; $F_{(4,\ 115)} = 1.799$, $P > 0.05$). Sucrose dominated the sugar composition, accounting for almost 97% of the total sugars. The less abundant sugars in EFN found were glucose (ca. 1.7%), fructose (ca. 0.9%) and traces of manitol, sorbitol and trehalose (Table 5.1). Amongst amino acids, threonine and glutamine were the highest in all varieties tested. Glutamate and arginine were also abundant (Table 5.2).

Table 5.1: Mean ± SE (μg/mg of EFN) of sugar constituents in the extrafloral nectar collected from five varieties of *B. juncea* plants *viz varuna*, *P. bold*, *P. bahar*, *P. agarni* and *JDG*. ND: not detected

	Varuna	*P. bold*	*P. bahar*	*P. agarni*	*JDG*
Sorbitol	0.01 ± 0.01	0.03 ± 0.02	ND	0.03 ± 0.03	ND
Manitol	0.26 ± 0.02	0.26 ± 0.02	0.27 ± 0.01	0.24 ± 0.03	0.29 ± 0.01
Trehalose	ND	0.03 ± 0.02	ND	0.03 ± 0.02	ND
Glucose	5.80 ± 0.95	11.33 ± 2.87	12.17 ± 1.90	8.62 ± 1.66	8.73 ± 1.47
Fructose	2.86 ± 0.74	6.33 ± 1.81	5.95 ± 1.62	4.63 ± 1.18	5.34 ± 1.26
Sucrose	528.81 ± 14.77	550.24 ± 13.62	533.49 ± 9.21	488.43 ± 22.17	557.56 ± 4.74
Total sugars	537.73 ± 14.87	568.21 ± 14.02	551.89 ± 9.75	501.97 ± 22.67	571.92 ± 5.03

In contrast to sugars, the concentration of total amino acids differed significantly between varieties (Kruskal- Wallis test; $H_4 = 12.295$, $P = 0.015$). Additionally, we analyzed glucosinolates, which are the main secondary compounds in Brassicaceae, in *Pusa bold*, *P. bahar*, *P. agarni* and *JDG*. The total concentration of glucosinolates was about 2.42 ± 0.25 μmol/mg of EFN and was similar in all four varieties (Univariate ANOVA; $F_{(3,\ 8)} = 8.44$, $P > 0.05$). Gluconapin (ca. 75%) and sinigrin (ca. 22%), with traces of glucobrassicanapin and gluconasturtiin, were the main constituents of glucosinolates in EFN.

Table 5.2: Mean ± SE (μM) of amino acid constituents in the extrafloral nectar collected from five varieties of *Brassica juncea* plants *viz. varuna*, *P. bold*, *P. bahar*, *P. agarni* and *JDG*. ND: not detected

	Varuna	*P. bold*	*P. bahar*	*P. agarni*	*JDG*
Arginine	25.32 ± 3.91	41.02 ± 5.48	33.41 ± 5.44	64.25 ± 9.27	29.507 ± 3.50
Lysine	7.70 ± 1.33	17.45 ± 2.46	17.06 ± 3.16	25.67 ± 3.94	15.72 ± 2.23
Glutamine	29.95 ± 3.76	51.58 ± 6.28	62.54 ± 7.18	90.48 ± 11.04	53.75 ± 6.81
Asparigine	9.23 ± 1.75	21.23 ± 3.01	22.41 ± 3.89	34.81 ± 5.24	22.56 ± 3.02
Alanine	11.77 ± 2.35	22.24 ± 3.33	16.41 ± 2.81	32.39 ± 4.21	19.84 ± 2.94
Threonine	24.23 ± 3.89	75.52 ± 13.04	94.97 ± 18.11	140.99 ± 37.04	64.02 ± 7.95
Glycine	0.65 ± 0.22	0.74 ± 0.15	0.81 ± 0.20	26.07 ± 24.50	0.70 ± 0.15
Valine	12.13 ± 2.13	23.60 ± 3.25	23.00 ± 4.03	38.09 ± 5.32	22.71 ± 3.08
Serine	14.72 ± 2.57	27.64 ± 3.97	26.73 ± 4.36	46.66 ± 6.08	30.31 ± 4.13
Proline	15.97 ± 3.86	30.11 ± 3.83	26.67 ± 4.79	50.40 ± 6.56	20.17 ± 3.15
Isoleucine	ND	ND	ND	ND	ND
Leucine	1.62 ± 0.34	3.36 ± 0.65	5.21 ± 1.08	3.23 ± 0.73	4.96 ± 0.71
Methionine	0.64 ± 0.15	1.27 ± 0.20	1.67 ± 0.33	1.86 ± 0.30	1.61 ± 0.29
Histidine	4.10 ± 0.71	8.70 ± 1.19	8.69 ± 1.45	12.28 ± 1.61	8.23 ± 1.11
Phenylalanine	4.45 ± 0.81	7.79 ± 1.13	7.28 ± 1.20	11.99 ± 1.56	7.90 ± 1.15
Glutamate	17.51 ± 3.35	46.33 ± 6.57	48.22 ± 7.76	69.68 ± 9.07	43.04 ± 6.03
Aspartate	6.21 ± 1.14	12.39 ± 1.92	15.95 ± 2.72	27.69 ± 3.61	14.10 ± 2.03
Cystine	0.01 ± 0.01	ND	ND	0.02 ± 0.02	ND
Tyrosine	3.75 ± 0.62	6.13 ± 0.86	6.34 ± 1.04	8.89 ± 1.23	6.24 ± 0.85
Tryptophan	1.09 ± 0.20	1.99 ± 0.31	1.80 ± 0.34	2.41 ± 0.38	1.77 ± 0.27
Total AA	191.05 ± 66.81	399.12 ± 50.43	419.16 ± 84.94	687.88 ± 115.29	367.15 ± 45.56

Time course of EFN induction after generalist damage

Nectar was collected repeatedly at 8 h-intervals for the first 48 h to determine the temporal dynamics of nectar secretion in plants damaged with the leaf-chewing generalist *S. exigua* larvae and undamaged plants. Thereafter, because of decreased EFN production, the sampling was continued every 16 h, until EFN production ceased. A repeated measures ANOVA determined that average mass of EFN (μg) secreted per plant differed significantly over time (Greenhouse-Geisser corrected, Time, $F_{(1.549, 9.291)} = 7.867$, $P = 0.013$). This was mainly due to a decrease in nectar secretion at later time points and not due to any particular diurnal pattern in EFN production (Figure 5.4a).

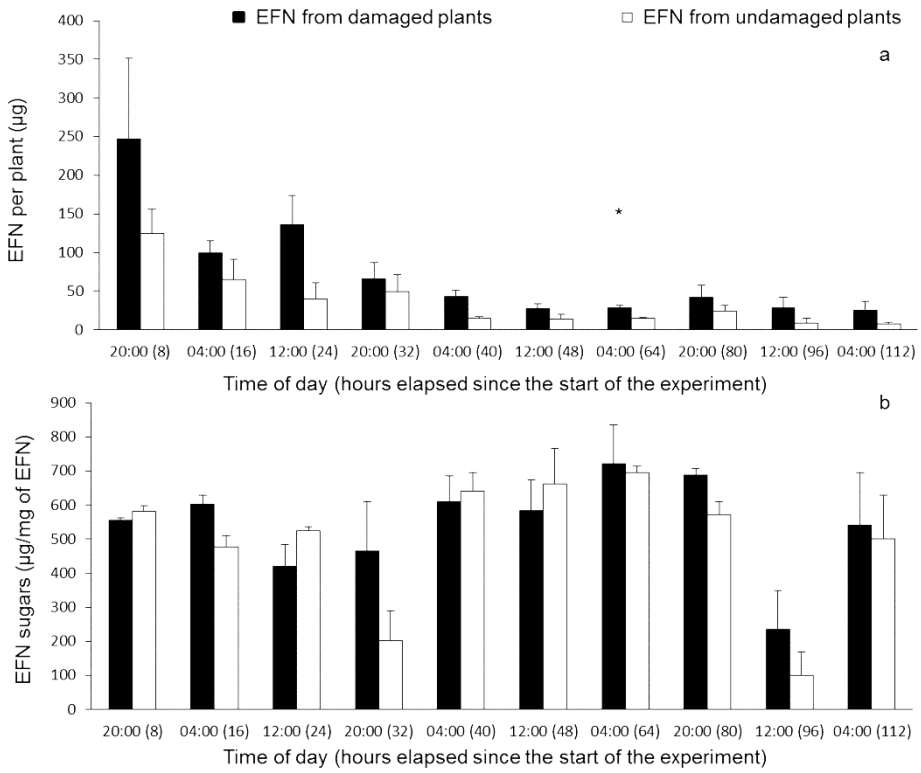

Figure 5.4: Temporal induction of (a) EFN (µg) and (b) sugars (mg/g of EFN) per plant of *Brassica juncea* (n= 20) after damage by *Spodoptera exigua*. Asterisks indicate significant induction at individual time points due to damage (independent sample *t*- test; P< 0.05).

Even though EFN production was higher in damaged plants at individual time points [40 h (uncorrected independent sample *t*-test; t_6 = 3.425, P = 0.031); 64 h (t_6 = 4.950, P = 0.003)], there was no overall statistical difference between the time points due to damage (Greenhouse-Geisser corrected, Damage, $F_{(1.549, 9.291)}$ = 1.037, P = 0.372). Overall there was no significant change in EFN sugar concentration due to damage (Greenhouse-Geisser corrected, Damage, $F_{(3.169, 19.016)}$ = 1.318, P> 0.05), despite significant differences at individual time points [16 h (uncorrected independent sample *t*-test; t_6 = 2.984, P = 0.025); 80 h (t_6 = 2.809, P = 0.031)] (Figure 5.4b).

Induction by herbivores of different feeding guilds

The amount of EFN produced was compared between intact plants and plants damaged by different above- or belowground herbivores every 24 h for five consecutive days. Since this experiment was conducted in different environmental conditions, damage by *S. exigua* was repeated to verify the consistency of induction. A repeated measure ANOVA revealed that aboveground damage by leaf-chewing specialist *P. brassicae* larvae ($F_{(4, 16)}$ = 4.030, P = 0.019) or the generalist aphid *M. persicae* (ANOVA, $F_{(4, 16)}$ = 5.308, P = 0.006) as well as root feeding specialist *D. radicum* larvae ($F_{(4, 16)}$ = 3.856, P = 0.022) increased EFN production over time. When analyzed for individual days, *P. brassicae* induced EFN on two days [day 1 (uncorrected independent sample *t*-test; t_6 = -5.500, P = 0.005); day 3 (t_6 = -3.500, P = 0.025)]. EFN production increased on three days following damage by *M. persicae* [day 2 (t_6 = -4.914, P = 0.008); day 3 (t_6 = -5.000, P = 0.007); day 4 (t_6 = -3.536, P = 0.024)]. Interestingly, EFN was not induced by *D. radicum* at individual time points. When damaged by the leaf-chewing generalist *S. exigua*, EFN was not significantly induced over the period of study, although a significant effect of damage was seen on day 1 (uncorrected independent sample *t*-test; t_6 = -0.400, P = 0.016). In addition, for *M. persicae* ($F_{(4, 16)}$ = 7.205, P = 0.001) there was an interaction between day and damage. This interaction was not significant in the other insects studied (Figure 5.5).

When a choice between water, EFN and sucrose was presented to the parasitoids of each of the tested herbivores, a significant preference of EFN and sugar over water was shown by *C. glomerata,* the parasitoid of *P. brassicae* [$X^2_{(2)}$ = 11.511, P = 0.003] and *T. rapae,* the parasitoid of *D. radicum* [$X^2_{(2)}$ = 9.056, P = 0.011]. A trend for EFN preference was shown by *D. rapae*, the parasitoid of *M. persicae*, but this preference was not significant [$X^2_{(2)}$ = 5.286, P = 0.071]. *Cotesia marginiventris,* the parasitoid of *S. exigua*, did not show any preference for the solutions offered [$X^2_{(2)}$ = 0.483, P = 0.786] (Figure 5.6).

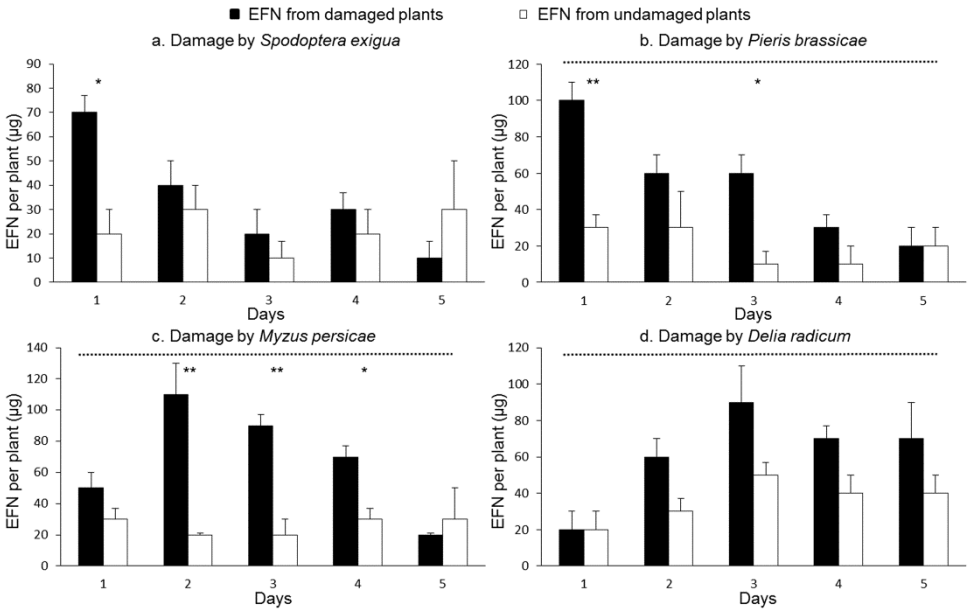

Figure 5.5: Temporal induction of EFN (µg) per plant of *Brassica juncea* (n= 15) after damage by insects of different feeding guilds. A horizontal line above the graph indicates a significant induction over time (repeated measures ANOVA) due to damage by (a) *Spodoptera exigua* (P > 0.05) (b) *Pieris brassicae* (P = 0.001) (c) *Myzus persicae* (P = 0.013) (d) *Delia radicum* (P = 0.017). Asterisks indicate significant induction at individual time points due to damage (independent sample *t*- test; *P < 0.05; **P < 0.01).

Parasitoid survival and fecundity

Survival of *C. marginiventris* (Survival analysis, $X^2_{(2)}$ = 23.274, P < 0.001), *C. glomerata* ($X^2_{(2)}$ = 22.914, P< 0.001) and *Di. rapae* ($X^2_{(2)}$ = 11.779, P< 0.005) was affected by diet. The survival of *T. rapae*, on the other hand, was not affected ($X^2_{(2)}$ = 1.329, P = 0.5144). The survival rates of *C. marginiventris* females showed significant differences in the pairwise comparisons between EFN, sucrose and water (Log-rank test, P< 0.001). On the other hand, females survived equally well on EFN and sucrose in the case of *C. glomerata* (P = 0.5073) and *Di. rapae* (P = 0.699), but both performed significantly better on EFN and sucrose than on water (P< 0.0001) (Figure 5.7).

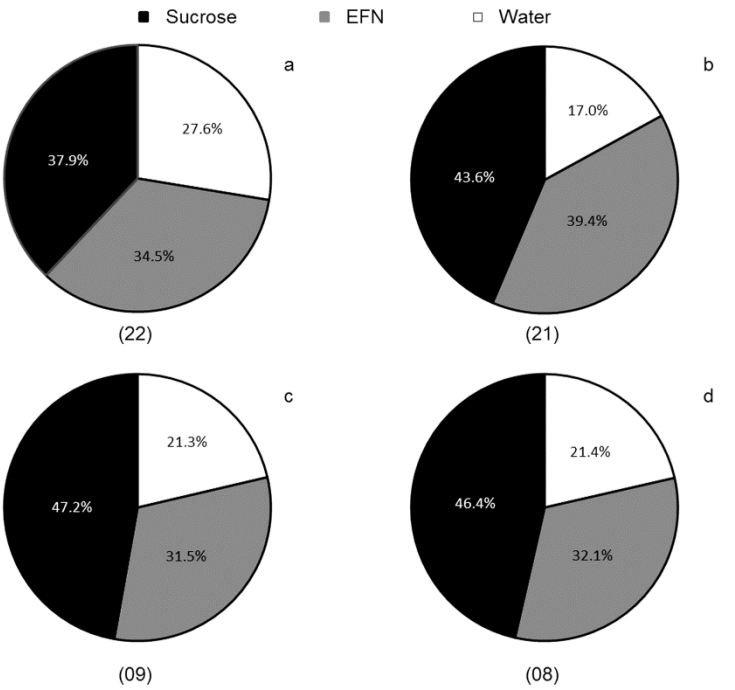

Figure 5.6: Orientation response of naive females of (a) *Cotesia marginiventris* (n= 29) (b) *C. glomerata* (n= 94) (c) *Trybliographa rapae* (n= 89) (d) *Diaeretiella rapae* (n= 56) when offered a choice between water, sucrose and EFN. Preference was significant for *C. glomerata* (chi-square test; P = 0.011) and *T. rapae* (P = 0.003). Numbers in parentheses represent the number of females which did not make a decision and hence were excluded from calculations.

The fecundity of two of these parasitoids (n = 15), *viz.*, *C. marginiventris* and *T. rapae*, was determined after they died in the survival experiment. In *C. marginiventris*, the number of eggs in the ovaries were significantly higher when fed on EFN (166.80 ± 1.73) or sucrose (168.27 ± 0.89) on the first day after emergence than in those fed only on water their whole life (156.6 ± 1.88; ANOVA; $F_{(2,42)}$ = 16.337, P< 0.001, Bonferroni post-hoc analysis, sucrose versus water, and EFN versus water both P < 0.001). We found a similar trend in *T. rapae* with egg loads higher for females that were offered sucrose (99.60 ± 0.98) or EFN (94.00 ± 2.07) and as compared to those offered water (91.13 ± 2.61; ANOVA; $F_{(2,42)}$ = 4.597, P< 0.05, Bonferroni post-hoc analysis, sucrose versus water, P = 0.014).

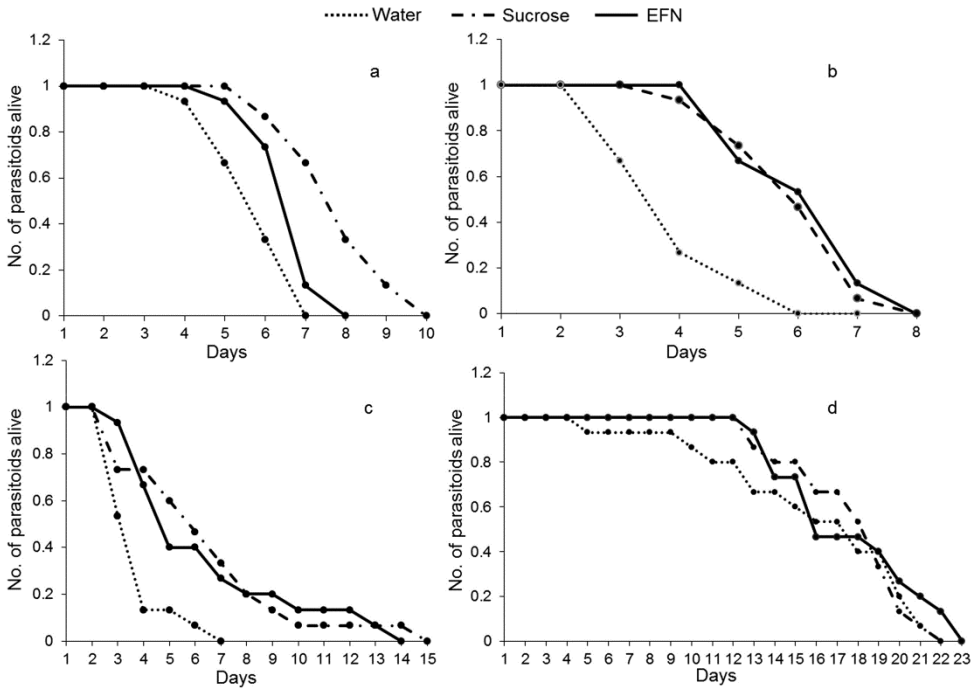

Figure 5.7: Survival curves of parasitoids (n= 15) when offered 10 μL of water, sucrose or EFN for the first 24h after emergence. Data was analysed using survival analysis (a) *Cotesia marginiventris* (*P*< 0.001), (b) *C. glomerata* (*P*< 0.001), (c) *Diaeretiella rapae* (*P*< 0.005), (d) *Trybliographa rapae* (*P* = 0.5144).

Discussion

Our study describes the occurrence of EFN in *B. juncea*. The family Brassicaceae, to which *B. juncea* belongs, has not been known to possess extrafloral nectaries before (Keeler 2008). These nectaries are found on the stem, peduncles and in leaf axils, and secrete nectar that predominantly contains sugars. Ultrastructural studies showed that these nectaries are formed as pocket-like structures in the epidermis of the stem only just before the onset of flowering. Feeding activities of three of the four above- and belowground herbivores tested enhanced the amount of secreted nectar. Parasitoid arrestment, survival and fecundity were enhanced by EFN, thus suggesting a possible ecological role of the EFN as an indirect defense.

The closest relative of Brassicaceae that possess extrafloral nectaries is *Capparis retusa* (Order Brassicales, family Capparaceae), where the nectaries look like umbilicated glands containing a specialized parenchyma vascularized with xylem and phloem (Di Sapio *et al.* 2001). Moreover, floral nectaries in many species of Brassicaceae have been described as protuberant gland structures surrounded by a modified stomata, which maintain a firm contact between cells and subjacent parenchyma cells (Davis *et al.* 1986, Davis *et al.* 1994, Davis *et al.* 1998, Baum *et al.* 2001). In contrast, the structure of extrafloral nectaries in *B. juncea* is a pocket-like cavity beneath the modified stomata (Figure 3a and b). A few species like *Citrus limon* (Rutaceae) and *Sambucus nigra* (Adoxaceae) are known to possess nectaries with a pocket-like cavity (Fahn 1979, Fahn 1988), but such nectaries have not been described in the order Brassicales (Elias 1983, Nepi 2007, Keeler 2008). The modified stomata surrounding the extrafloral nectaries in *B. juncea* are common in most floral and extrafloral nectaries. These stomata are always open and hence do not regulate nectar secretion (Elias 1983, Bernardello 2007, Nepi 2007, Heil 2011, Escalante-Pérez and Heil 2012). The formation of pocket- like cavities in *B. juncea* could either be by cell lysis or by modification of the substomatal chamber. Future work in this direction could provide better insight into the ontogeny of these cavities.

Previous studies have demonstrated that EFN may either show a diurnal pattern by an increase in secretion at certain day times (Heil *et al.* 2000), or a constant secretion irrespective of the time of the day (Bentley 1976). We found the nectaries in *B. juncea* secreted small amounts of nectar throughout the day and there was no diurnal rhythm. However, in separate studies, plants failed to produce nectar in climate cabinets with lower light intensity or quality. The importance of light intensity was confirmed by the fact that these plants began to produce EFN when moved from the growth cabinets to the greenhouse (V. Mathur and N. M. van Dam, unpublished data). Thus, although no diurnal pattern was observed, light quality could play a significant role in EFN production.

We found that EFN secretion began at the flowering stage and continued till silique formation. The flowering period is known to be a critical period and plants need to be defended the most at this stage to protect their reproductive organs. Enhanced production of EFN has often been reported to enhance plant defense by recruiting ants and other natural enemies of herbivores to plants (Bentley 1977, Rudgers 2004, Yamashiro and Yamashiro 2008). EFN droplets were observed in high amounts on the peduncles of the plant, thus strengthening the idea that they are specifically supporting the defense of flowers and seed production. This pattern is in accordance with the spatio-temporal distribution of defense investment as predicted by the optimal defense theory (McKey 1974, McKey 1979, Rhoades 1979).

EFN is known to attract and sustain ants, parasitoids and predators, and thus serves as an indirect defense against herbivores (Koptur 1992, Heil 2008). Carbohydrates and free amino acids in the nectar are important for their attraction. Floral and extrafloral nectar sugar concentrations range from 8% to 80% depending on the plant species, but may also vary with environmental conditions (Nicolson and Nepi 2005, Nicolson and Thornburg 2007, Pacini and Nepi 2007). EFN produced by *B. juncea* was viscous and around 50% of the total weight of EFN was sugars in all the varieties examined (Table 1). This concentration is similar to that observed for its floral nectar (Masierowska 2003). Sucrose was the main sugar present in *B. juncea* EFN (ca. 97% of the total sugars). It is well known that sucrose is the preferred carbohydrate for carbon transfer in the phloem and 95% of total sugars in phloem sap are sucrose (Yeo and Flowers 2007). In Brassicaceae, floral nectar is richer in hexoses, indicating that processing of the phloem-transported sugars takes place before it is excreted (Percival 1961, Baker and Baker 1983). However, EFN in *B. juncea* predominantly contained sucrose, and therefore we assume that the nectar is being directly secreted from phloem, despite the lack of visual connections between phloem and nectaries in the transverse sections.

In addition to sugars, amino acids may also play an important role in the attractiveness of nectar, even when they are about 100 to 1,000 times less concentrated than sugars (Heil 2011). Amino acids have been reported in foliar nectar of pteridophytes, as well as floral and extrafloral nectar of angiosperms. Serine, glycine, alanine, arginine and proline are typically abundant in these secretions (Baker and Baker 1983, Pate *et al.* 1985). However, in the EFN of *B. juncea*, threonine, glutamine, arginine and glutamate were the most abundant amino acids. Besides sugars and amino acids, other compounds like volatiles, proteins, lipids and several classes of secondary metabolites have been reported in nectar (Kessler and Baldwin 2007, Gonzalez-Teuber and Heil 2009). In our study, the EFN in *B. juncea* contained glucosinolates, which are well known secondary compounds in Brassicaceae, conferring resistance not only insects (Hopkins *et al.* 2009), but also microbes (Bressan *et al.* 2009). The glucosinolate profile was found to be essentially similar to that found in the earlier studies in the leaves of *B. juncea* (Mathur *et al*. 2011). Possibly glucosinolates aid in keeping the nectar sterile, but they may also prevent the entry of phytopathogens from the permanently opened nectary pores (Heil 2011).

Plants increase EFN production as an indirect defense when they are damaged by root or shoot herbivores (Wackers and Bezemer 2003, Heil 2004, Escalante-Pérez and Heil 2012). EFN production in *B. juncea* was also found to be influenced by herbivory. Nectar secretion was increased by chewing above- and belowground herbivores, but also by sap sucking herbivores. However, this induction varied temporally with the feeding insect species. Most of the earlier studies have been done in the context of ant–plant interactions (Bentley 1976, Heil *et al.* 2001, Bixenmann *et al.* 2011), but several studies have also addressed the role of EFN in the attraction of parasitoids (Stapel *et al.* 1997, Lewis *et al.* 1998, Rose *et al.* 2006). For a high reproductive success, disruption of parasitoid's host foraging process should be minimal so that most of its time and energy can be allocated to finding hosts and producing offspring. In our study, *C. glomerata* and *T. rapae* showed a significant preference for EFN and *Di. rapae* showed a similar trend, emphasizing the possible importance of EFN in parasitoid attraction and

retention in *B. juncea* plants. In contrast, the overall preference of *C. marginiventris* to either of the choices offered was not observed. This lack of statistically significant response may be due to low number of responding parasitoids. Resource finding in parasitoids is typically interplay between an attractant stimulus, that causes them to orientate towards the source, and an arrestant stimulus due to which they reduce/stop searching behavior (Dethier *et al.* 1960). Here, we observed that the orientation of parasitoids was more associated with arrestment than attraction. The assumption also arises from our finding that parasitoids were equally attracted to EFN and sucrose solution. It is known that experienced parasitoids associate the perceived odor with the presence of hosts (Vet *et al.* 1995, Turlings and Wäckers 2004). However, whether parasitoids can perceive EFN from distance is still unknown (Rose *et al*. 2006).

By secreting EFN, plants provide parasitoids a rich carbohydrate resource on the plant itself, and thus an opportunity to live and forage longer. In our survival analysis, three of the tested parasitoids survived significantly longer when fed on EFN or sucrose just for one day, as compared to parasitoids that were given water only. Longevity is directly proportional to the quality of carbohydrate consumed by the parasitoid (Wackers 2001, Giron *et al.* 2004). Our study suggests that EFN in *B. juncea* may act as a good carbohydrate resource for parasitoids. Interestingly, the glucosinolates in EFN only seemed to have a significant effect on the survival of the generalist parasitoid *C. marginiventris*, which survived longer on sugar water than on EFN (Figure 5.7a). Specialist parasitoids of specialist Brassica herbivores were not affected by glucosinolates in the EFN, which may reflect their adaptation to these typical *Brassica* defenses that may also be present in their herbivorous hosts (Hopkins *et al*. 2009). Additionally, the survival rate of *T. rapae* was similar on all three treatments. *T. rapae*, being a pro-ovigenic parasitoid, is known to reabsorb its eggs to fulfill its nutritional requirements and live longer under low nutrient conditions (Jervis *et al.* 2001). However, previous field studies have found *T. rapae* lived longer when provided with floral nectar throughout their lives (Nilsson *et al.* 2011). On examination of the ovaries of *C. marginiventris* and *T. rapae* after the survival experiment, we found that the potential fecundity of both

parasitoids was higher after feeding on EFN on the first day than when they were given only water. This suggests that EFN was a nutritious food source for these parasitoids. Our study thus demonstrates that parasitoids may have a longer life span and a higher fecundity when feeding on EFN.

Based on the hypotheses regarding the evolution of (floral) nectar secretion proposed by de la Barrera and Nobel (2004), there are two plausible mechanisms explaining the origin of the amorphic extrafloral nectaries in *B. juncea*: the leaky phloem hypothesis and the sugar excretion hypothesis. According to the first, nectar secretion may have originated as leakage of phloem solution, resulting from the structural weakness of developing tissues exposed to high pressure in the phloem. The sugar excretion hypothesis suggests that the solute accumulation resulting from the relatively high transpiration rates of flowers results in nectar secretion to dispose of excess solutes (de la Barrera and Nobel 2004). Since extrafloral nectaries observed in *B. juncea* are initially amorphic, we suggest that they are in the early stages of evolution. Moreover, the nectar was more abundant in sucrose, as in phloem, than in hexose sugars as in its floral nectar. Therefore, we speculate that the leaky phloem hypothesis of the origin of nectar can be applied to these nectaries. However, the physiological mechanism by which this is achieved needs to be investigated before drawing a final conclusion.

The development of extrafloral nectaries in *B. juncea* may have a role in the tritrophic interaction among plants, herbivores and parasitoids. With this study, we described the presence of EFN in *B. juncea*, a novel insight in the defense strategies employed by Brassicaceae plant species. We showed that the nectar is inducible and that it could be used by several natural enemies of the main herbivore species of this plant both above- and belowground. Further studies are required to understand the evolution and ecological significance of these extrafloral nectaries as indirect defenses.This knowledge will contribute to the development of better biological control practices and hence more sustainable agricultural practices for *Brassica* crops.

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Chapter 6

Discussion and synthesis

Picture by: Sonia Dourlot

Vartika Mathur

In numerous natural and agricultural systems, attack by herbivores and pathogens has been shown to induce plant responses. There is ample evidence that plants can perceive diverse stimuli from insect oviposition and feeding, including wounding patterns and insect-derived elicitors in saliva, and adapt their responses accordingly (Agrawal 2000, Hilker and Meiners 2002, Van Zandt and Agrawal 2004, De Vos *et al.* 2005, Viswanathan *et al.* 2005). Additionally, studies on the temporal and spatial scale of induced responses have extended our understanding of the detailed mechanisms behind this phenomenon (van Dam and Raaijmakers 2006, Travers-Martin and Müller 2007, Bruinsma *et al.* 2009, Gomez *et al.* 2010). The temporal and spatial variations in induced responses make plants more heterogeneous, and possibly more difficult for the herbivore to cope with (Karban *et al.* 1999, Orians and Jones 2001, Steinbrenner *et al.* 2011). Therefore, timing is critical in induced plant resistance.

Herbivore-induced responses are specific and dynamic

The aim of my study was to assess the timing and synchronization of different herbivore-induced plant responses in *Brassica juncea*, and how this affects the herbivores and their parasitoids. In Chapter 2, I measured the induction of primary (sugars and amino acids) and secondary compounds (glucosinolates) as well as structural defences (leaf size and trichomes) over two weeks following herbivory by generalist caterpillars of different *Spodoptera* species in the Netherlands and India. I hypothesized that these responses vary in time. My results showed that the various traits were induced at the same temporal scale in the Netherlands and India despite variation in environmental conditions and herbivore species. This indicates that the timing and occurrence of induced plant responses in *B. juncea* are robust traits.

However, the degree to which induced responses in *B. juncea* are specific to different herbivores was still poorly understood. To asses this specificity, I compared induced plant responses to two different leaf-chewing caterpillars. In Chapter 3, I measured glucosinolates, trichomes and leaf size over a period of

twenty days after the plants were damaged by the specialist *Plutella xylostella* alone as well as in combination with the generalist *S. exigua*. From the combined analysis of Chapter 2 and Chapter 3, I concluded that glucosinolates, the representative secondary metabolites of the family Brassicaceae, increased from the fourth day after damage by the generalist *Spodoptera* spp. alone or in combination with *P. xylostella.* They remained at higher levels in damaged plants throughout the study period. The intensity of glucosinolate induction was, however, higher when the plants were damaged by double herbivory. When the plants were damaged by the specialist *P. xylostella* alone, induction of glucosinolates was delayed until 14 days following damage and elevated to significant levels only after 20 days from plant damage. The increase in glucosinolate levels is mainly caused by changes in alkenyl glucosinolate concentrations, which constitute 97% of total glucosinolates. On the other hand, indole glucosinolate concentrations were not affected by the generalist or by double herbivory, but significantly declined following herbivory by the specialist.

Trichome induction also varied in time with respect to damage either by generalist, specialist or double herbivory. The density of trichomes increased significantly from seven days after herbivory by the generalist. This increase was quicker when the plants were damaged by the specialist alone or simultaneously with the generalist, reaching significantly higher levels from the fourth day onwards. Trichomes remained at higher levels throughout the study period and showed similar temporal induction patterns in all the damage treatments on both adaxial and abaxial surfaces.

These combined analyses of temporal changes in structural and chemical traits in response to different herbivores therefore support my assumption that plants have the ability to distinguish between different types of biotic challenges and respond specifically to each of them. In general, I found that the systemic induction of glucosinolates was only slightly faster than that of trichomes when the plants were damaged by the generalist, and reached the highest levels at the same time in case of double herbivory. Moreover, trichome induction was faster

when the plants were damaged by the specialist whereas glucosinolate induction was delayed. These results thus contradict my hypothesis that the induction of structural defences in plants is always slower than chemical induction.

"One man's meat is another man's poison"

Whether specificity of wound-induced responses facilitates or impedes the establishment of an insect and to what extent, strongly depends on the plant and the insect in question. For example, a plethora of studies has demonstrated that generalist herbivores are more susceptible to induced plant responses than specialist herbivores (Karban and Baldwin 1997, Agrawal and Sherriffs 2001, Travers-Martin and Müller 2007, Poelman *et al.* 2008, van Dam and Oomen 2008). However, this general view does not always apply. For example, Agrawal (2000) not only provides an excellent example of this contradiction, but also establishes the importance of specificity in induced responses by demonstrating that induced responses in wild radish are specific to each of four species of leaf-chewing herbivores examined and may confer specific resistance against later arriving herbivores.

Other studies also reveal that induced responses may also provide resistance against specialist herbivores (van Dam *et al.* 2000, Traw and Dawson 2002, Chung and Felton 2011). One of the reasons for this contradiction could be that induced responses are dynamic responses and their temporal duration may have important consequences for their effectiveness, although limited information is available for this assumption (Karban 2011). Hence, induced responses should not be customarily considered as induced resistance. Instead, it must be first observed whether induced responses provide resistance to plants at all, by comparing the performance of both generalist as well as specialist herbivores on undamaged plants and damaged plants on a relevant temporal scale.

To test the specificity of the effect that induced responses have on herbivores, I investigated the preference and performance of *S. litura* and *P. xylostella* after *B. juncea* was damaged at different time points by generalist, specialist or combined

herbivory by the same two pest species (Chapter 2 and 3). I found that in all treatments, the generalist *S. litura* always preferred and essentially performed better on undamaged plants as compared to damaged plants at different time points. However, the degree of avoidance and performance among damaged plants varied with treatment and time elapsed after herbivore attack. The larvae of this generalist gained the least weight when they were fed on leaves on the ninth day after the plant was damaged by a conspecific (Chapter 2). Notably, they even lost weight when their host plants were damaged by a specialist 10 or 20 days earlier (Chapter 3). The specialist *P. xylostella*, in contrast, always preferred the damaged plants. However, like *S. litura*, the degree of preference and performance varied with time elapsed after the plant was damaged. *P. xylostella* larvae preferred to feed and performed better on plants damaged between one and two weeks earlier by a generalist or a conspecific (Chapter 2, 3). As opposed to the generalist, they preferred and performed the least on the undamaged plants. My results show that the generalist *S. litura* is more sensitive to induced responses in *B. juncea* than the specialist *P. xylostella*. My studies thus agree with the hypothesis that plants induce specific responses to damage by generalists and specialists and that these responses have different effects on later arriving herbivores.

The strongest evidence for the effect of temporal dynamics on herbivore preference and performance could be observed in my study on double herbivory. When the plants were subjected to double herbivory, *S. litura* gained more weight on plants that were damaged four days earlier as compared to undamaged plants, but significantly lost weight when fed leaves on plants damaged 14 or 20 days earlier. Conversely, the weight gain of *P. xylostella* was the highest on plants damaged 10 days earlier, after which it decreased and almost corresponded to that of undamaged plants when they were fed with plants damaged 20 days earlier (Chapter 3).These observations clearly indicate that preference and performance of generalists and specialists are dependent on the time elapsed after damage, which shows their specificity in responding to

induction by earlier arriving herbivores. These studies thus validate my hypothesis that although induced responses essentially render plants more resistant to generalists and possibly more susceptible to specialists, the magnitude of the resistance and susceptibility is dependent on the temporal dynamics of the induced responses.

Plants recruit an army against attackers

In addition to direct defences, plants also rely on indirect defences by attracting the herbivore's natural enemies and/or providing them with food and shelter (Heil 2008). The most widely studied indirect defence is the herbivore induced production of volatile organic compounds (Dicke 1999, Paré and Tumlinson 1999, Holopainen 2004). The emission of volatiles following herbivory can be a passive release of stored compounds or an active production of existing or novel compounds. Consequently, the volatile blend emitted by damaged plants is dynamic and differs from that of intact plants (Vet 1999). In a 72 h-study of damaged-induced *B. juncea* volatile emissions (Chapter 4), I found an increase in the emission of isothiocyanates, green leaf volatiles (GLVs) and sulfides, besides some alcohols, aldehydes and ketones. These classes of compounds are known to increase following herbivory in Brassicaceae and other plant species (van Poecke *et al.* 2001, Kessler *et al.* 2006, Arimura *et al.* 2009). In contrast, monoterpenes were either reduced in quantity or were not affected by *S. exigua* damage. In addition, sesquiterpenes did not show any reaction to herbivory. My study corroborates earlier studies that found little or no induction of these terpenes in Brassicaceae (Blaakmeer *et al.* 1994, Geervliet *et al.* 1997), even though the emission of these compounds increases following herbivory in other plant families (Kant *et al.* 2004, Copolovici *et al.* 2011). On the other hand, two homoterpenes, *viz.*, (E,E)-4,8,12-trimethyl-1,3,7,11 tridecatetraene (TMTT) and <3E>-4,8-dimethyl-1,3,7-nonatriene (DMNT), were produced *de novo* after 24 h of damage. These compounds are often newly produced or increase in quantity following herbivore damage or treatment with elicitors (Turlings *et al.* 1990, van

Poecke, et al. 2001, Pierre *et al.* 2011). Each volatile fluctuated at its own temporal pace during the period of my study, thus resulting in a different volatile blend at each studied time point following herbivory.

Herbivore-induced plant volatiles (HIPVs) not only act as reliable cues for parasitoids and predators for locating herbivores, but are also known to affect herbivore behaviour (Dicke and Vet 1999, De Moraes *et al.* 2001, Gouinguené *et al.* 2003, Bukovinszky *et al.* 2005, Ibrahim *et al.* 2005, Halitschke *et al.* 2008, Erb *et al.* 2010). Additionally, they mediate interactions with undamaged plant parts, neighbouring plants and microorganisms (Takabayashi and Dicke 1996, Heil 1999, Heil and Ton 2008). However, plant volatile blends vary according to the time elapsed after damage, the developmental stage of the plant and the damaging herbivore (Sabelis *et al.* 2007). The dynamic and 'volatile' nature of this indirect response, therefore, substantially influences the insect communities, both above- and belowground (Dicke and Vet 1999, D'Alessandro and Turlings 2005, Poelman *et al.* 2011).

In Chapter 4, I demonstrated temporal variation in the preference of females of generalist and specialist herbivores, *viz. S. litura* and *P. xylostella* and their parasitoids *Cotesia marginiventris* and *C. plutellae* to *B. juncea* following herbivory. I found that *S. litura* favoured undamaged plants. In contrast, *P. xylostella* as well as both parasitoid species preferred damaged plants. However, the degree of their preference differed with time after damage. *P. xylostella* and its parasitoid, *C. plutellae*, were attracted to plants damaged for 72 h. Earlier studies have revealed that *P. xylostella* respond to higher levels of isothiocyanates (Pivnick *et al.* 1994), while *C. plutellae* is attracted to high levels of dimethyldisulfide and dimethyltrisulfide (Reddy *et al.* 2002). The emission of these compounds was the highest at 72 h after damage, thereby suggesting that these compounds act as strong reliable cues to the specialists to locate their respective hosts. *C. marginiventris*, on the other hand was attracted to plants damaged for 48 h. Since this is a generalist parasitoid, it is known to utilize more generalized cues, such as terpenoids and GLVs for its host location (Turlings *et al.*

1991). In our study, the GLVs 3-hexen-1-ol and acetaldehyde were emitted at the highest level from plants damaged for 48 h, and hence these compounds may have a role in the attraction of *C. marginiventris*.

It has been known for some time that, as opposed to generalist herbivores, specialist herbivores and parasitoids are better adapted to make use of induced volatiles as host-finding cues (Kessler and Baldwin 2001, Vuorinen *et al.* 2004, Ibrahim, *et al*. 2005, Heil 2008). Differential responses by different species of herbivores' enemies may mediate the degree to which they compete for the same host. Moreover, the extent to which these insects are able to discriminate among specific volatiles may depend on their own ability as well as the species and developmental stage of the damaging herbivore (Takabayashi *et al.* 1995, De Moraes *et al.* 1998, Gouinguene *et al.* 2003). For example, the vulnerability of *Heliothis subflexa* to its parasitoid *Cardiochiles nigriceps* changed even with the part of the plant that the herbivore was feeding upon (De Moraes and Mescher 2004). In contrast to this specificity, *Cotesia* spp. are equally attracted to volatile blends induced by their natural host and by the non-host, as long as the type of damage (e.g. leaf chewing) is the same (Vos *et al.* 2001, van Poecke *et al.* 2003). In accordance with this hypothesis, my study in Chapter 4 also demonstrated an increased attraction of *C. plutellae* to *S. exigua*-infested plants. In general, the effect of HIPVs on herbivores and their enemies can be very specific and dynamic due to the changing blends of volatiles that occur at different time points [Chapter 4; (Bruinsma *et al*. 2009)]. Based on my study, I conclude that HIPVs can serve as a reliable cue for both herbivores and their parasitoids and that the temporal dynamics of the emitted plant volatile blends plays a key role in regulating these interactions.

An inside-out analysis: Genetic regulation of induced resistance mechanisms

In the past decade, integrated ecological, biochemical and molecular biological approaches have yielded important progress in our understanding of induced

plant defences. With the help of this integrated approach, the loose ends regarding the specificity in plant responses are now being tied. Many genes have been identified that may be involved in herbivore-induced plant resistance (Reymond *et al.* 2000, Voelckel and Baldwin 2004, Schmidt *et al.* 2005). It is established that the 'tailoring' of these responses to particular herbivores likely results from crosstalk between different signalling pathways, mainly jasmonic acid (JA), salicylate (SA), ethylene (ET) and abscisic acid (ABA) (Baldwin *et al.* 2001, Kessler and Baldwin 2002). The induction or suppression of these signalling pathways varies significantly in both quantity and timing with each herbivore species that is feeding on the plant (De Vos *et al*. 2005).

In order to investigate the complexity and dynamics of induced responses, I studied the expression profiles of 14 genes involved in the production of different classes of volatile compounds, glucosinolates and signalling pathways. I examined the temporal expression of these genes at five time points from 6 h until 72 h of damage in local and systemic leaves of undamaged and herbivore-damaged *B. juncea*. I found that *S. exigua* induces the JA pathway throughout the study period in both damaged and systemic leaves. Leaf-chewing herbivores typically induce the JA pathway (Schmelz *et al.* 2003, De Vos *et al*. 2005, Chung *et al.* 2008). In contrast, Diezel *et al.* (2009) found that damage by *S. exigua* results in upregulation of the SA pathway and comparatively lower JA and ET bursts in *Nicotiana attenuata*. Earlier studies have also shown that the SA pathway suppresses the JA-pathway in *Arabidopsis* (Koornneef *et al.* 2008, Leon-Reyes *et al.* 2010). However, the gene expression profiles of genes in the JA, ET and SA pathways in Chapter 4 contradict the results of Diezel *et al*. (2009). In fact, I found that after herbivory by *S. exigua*, the SA pathway is down regulated in *B. juncea*. Additionally, the temporal gene expression patterns of JA and ET were similar in my studies, suggesting that these pathways are (up)regulated in concert after *S. exigua* feeding.

There is ample evidence of the role of herbivore oral secretions in activating the signalling pathways resulting in defence responses, such as volatiles and

glucosinolates (Alborn *et al.* 1997, Arimura *et al.* 2005, Schmelz *et al.* 2007, Hopkins *et al.* 2009). When damaged by *S. exigua*, I found a significant induction in the expression of aliphatic glucosinolate biosynthetic genes, whereas there was no effect on indole glucosinolate genes (Chapter 4). The gene expression studies aptly explain a steady increase in the emission of 1-butene-4-isothiocyanate, a volatile glucosinolate product of aliphatic glucosinolates (Chapter 4). They also correspond to the observation that *S. exigua* feeding significantly increases aliphatic glucosinolate levels and has no effect on the indole glucosinolates (Chapter 2). These studies corroborate studies by Bidart-Bouzat and Kliebenstein (2011) suggesting that aliphatic glucosinolates are induced by *S. exigua*, and this induction is also expressed at the gene level in *Arabidopsis thaliana*. Additionally, the GLV and sulfide genes were upregulated during most of the study period, which coincides with the steady increase in the emission of these compounds with time, after damage has started. None of the mono- and sesquiterpene synthesis genes showed any significant change in their expression, confirming my observation from the volatile analysis (Chapter 4). The signals in the systemic leaves were weaker as compared to local leaves, but their temporal dynamics was essentially similar. With these results, I validate my hypothesis that the temporal patterns in induced responses are mirrored in the dynamics of their gene expression profiles.

Sweetness in a spice

Plants are known to produce extrafloral nectar (EFN) as an indirect resistance strategy (Heil *et al.* 2000, Wackers and Bezemer 2003, Lach *et al.* 2009). Predators and parasitoids of a plant's herbivores are attracted by EFN as a carbohydrate-rich resource (Gonzalez-Teuber and Heil 2009, Heil 2011, Escalante-Pérez and Heil 2012) and in turn provide the plant protection against these herbivores. EFN is known to be produced in 114 plant families, but has never been reported to occur in the family Brassicaceae (Keeler 2008). In Chapter 5, I report, for the first time, the occurrence of EFN in *B. juncea*. The extrafloral nectaries are simple, as is

the case in most plants bearing extrafloral nectaries (Kirchoff and Kennedy 1985). They are 'gestaltless' (shapeless) and cannot be seen with the naked eye. However, microscopic observations revealed a pocket-like structure guarded by modified stomata, which are always open. Unlike leaf stomata, modified stomata are unable to control their pore aperture. However, they are important in maintaining a firm contact between cells and subjacent parenchyma cells (Davis *et al.* 1986, Davis *et al.* 1994, Davis *et al.* 1998, Baum *et al.* 2001). The pocket-like structure of the extrafloral nectaries is different from the floral nectaries of Brassicaceae, which are protuberant glands (Davis, *et al*. 1994). To optimize EFN production as an indirect defence strategy, optimal defence theory predicts that EFN may be located among plant parts in proportion to their value to plant fitness and probability of attack (Holland *et al.* 2009). In accordance with this theory, EFN in *B. juncea* were secreted from the stem, flowering stalk and leaf axils only during flowering until silique formation, when the plant needs to be defended the most.

EFN from *B. juncea* was found to be mainly composed of sugars, but also traces of amino acids and glucosinolates were found (Chapter 5). The presence ofsugars and amino acid indicates that EFN may act as a suitable food source for natural enemies of herbivores. The glucosinolates in the EFN may provide protection against the entry of phytopathogens into the plant through the open stomata (Heil 2011). Even though EFN secretion was light dependent, I did not find a diurnal rhythm for its secretion. EFN secretion increased when the plant was damaged by above- and belowground chewing herbivores and an aphid. However, the temporal dynamics of this induction was different for each herbivore, thereby emphasizing the specificity of this plant trait.

The importance of EFN as a possible indirect defence strategy is well established. Earlier studies have found that an increase in EFN secretion leads to a higher rate of visits by the herbivores' natural enemies, and in turn, a better defence for plants. Most of these studies are performed on ants, which are known to kill or remove herbivores from the plants (Bentley 1976, Rudgers 2004, Heil

2011, Holland *et al.* 2011), but other herbivore enemies, such as parasitoids (Stapel *et al.* 1997, Wäckers *et al.* 2001, Rose *et al.* 2006) also utilize EFN. The flowering period is known to be an important stage of plant, when they need to be defended the most in order to protect their reproductive organs. The fact that EFN production in *B. juncea* started during the flowering stage and large numbers of EFN droplets were observed on the peduncles of the plant corroborate this view. Moreover, in my study I also found that EFN production influences the arrestment of nectar-foraging parasitoids and is capable of sustaining them (Chapter 5). The production of EFN agrees with the spatio-temporal distribution of defence investment of the optimal defence theory (McKey 1974, McKey 1979, Rhoades 1979). Therefore, I showed that EFN may potentially act as an important food source for parasitoids thereby contributing to the indirect defence strategies of *B. juncea* and affecting the outcome of the interaction between plants and their herbivores.

'Time' to deliberate

Induced plant responses are now widely accepted as an effective and widespread resistance strategy. Yet several pieces of the process still remain poorly understood. Induction is influenced by an array of external factors such as the type of damage (e.g. leaf chewing or sap sucking) and the species and developmental stage of the herbivore damaging the plant. Moreover, factors including the timing, amount and location of damage also significantly influence these responses. This causes induced responses to be variable in time and space. This variability may lead to an alternation of enhanced resistance and susceptibility on an extended temporal scale, which may substantially alter the impact of these responses on herbivore populations. Conceptual models of induced resistance describe a lag between damage and initial induction, followed by an increase in resistance to a peak and, in the absence of further damage, subsequent decay over a period of time (Schultz 1988). This model has been theoretically (Schultz 1988, Dobson and Crawley 1994), mathematically or

experimentally validated in terms of the population dynamics of the visiting herbivores and their natural enemies (Poelman, *et al*. 2008, Jansen *et al.* 2009). The effects of temporal dynamics have also been demonstrated in studies using hormones that temporally manipulate the signalling pathways (Adie *et al.* 2007, Koornneef, *et al*. 2008). However, most studies demonstrate the induction of plant responses either at one time point or only for a short period of time. Consequently, the interpretation of induced responses may seem like an incomplete jigsaw puzzle and raises questions regarding the ecological perspective (Traw and Dawson 2002, van Dam *et al.* 2004, Rodriguez-Saona *et al.* 2005, Travers-Martin and Müller 2007). My studies establish the importance of temporal dynamics in the ecological functions of induced responses. I found that these responses are temporally specific according to the species of the damaging herbivore and the number of species feeding on the plant simultaneously. This specificity of responses affects further interactions between the plant, herbivores and their natural enemies, which may in turn influence plant fitness. Furthermore, in my studies, I demonstrated that generalist herbivores are very sensitive to the temporal effects of induced responses. Hence, natural resistance of plants may play a major role in their control in agricultural fields. Therefore additional pest management strategies may be developed primarily to reduce damage by specialist herbivores.

Furthermore, temporal variation in induced responses may explain seemingly opposite results of earlier studies conducted using the same plant-insect models. For example, no change in the aliphatic glucosinolate levels was observed after 7 days of damage by the herbivore beetle *Psylliodes chrysocephala* on oilseed rape (Bartlet *et al.* 1999), whereas these levels were found to decrease after 10 days (Koritsas *et al.* 1991). However, a few studies have well demonstrated that timing of induction plays a significant role in herbivore resistance (van Dam and Raaijmakers 2006, Soler *et al.* 2007, Bruinsma *et al*. 2009). The studies presented in this thesis may thus be considered as a missing piece of the puzzle that would give a better insight in the dynamic nature of

induced resistance mechanisms. Therefore, my results will not only increase our fundamental ecological understanding of the temporal dynamics of various induced changes and its effects on the interacting species, but will also permit us to manipulate these interactions to our advantage, especially in agriculture.

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Insect-plant interactions are highly dynamic. For their protection, plants produce various structures and compounds which influence the behaviour, growth and/or survival of herbivores. Some of these compounds and structures are continuously expressed, even when the plant is not in danger. Such background defences are called constitutive defences. However, once an attack by insects or pathogens has occurred, plants may increase the concentration of existing defence compounds and/or start the production of novel structures and chemicals. This is known as an induced response.

Induced responses usually peak only for a certain period of time and each individual response may have its own temporal dynamics. The timing and localisation of each response is strongly correlated with its primary function. For example, wound-healing responses are quick. Alternatively, some compounds require the activation of biosynthetic pathways, which in turn involves the activation of hormonal signalling, gene expression and protein synthesis before these compounds can be produced. Consequently, there is a delay before the induced phenotype is formed and affects herbivores and their natural enemies. Therefore, determining the time interval between various changes occurring in the plant as well as their coordination and function in time is essential to understand the role of each induced response in direct or indirect resistance.

The study in this thesis essentially analyses the importance of temporal dynamics of various direct and indirect induced responses at the molecular, physiological and ecological level in *Brassica juncea* (Indian/Brown mustard, Brassicaceae). *B. juncea* was selected as a model for these studies because it belongs to family Brassicaceae which contains many economically important crops as well as *Arabidopsis*, which is a model plant for molecular, physiological and genetic studies. Additionally, the interactions of these plants with generalist and specialist herbivores and the resistance traits against these herbivores have been well studied. The research mainly focuses on: (a) detailing the timing of induced responses at the morphological, biochemical and molecular level in *B. juncea* following insect damage; (b) the ecological effects of direct and indirect

induced resistance at different time points by examining generalist and specialist insect behaviour. The main hypothesis of this thesis is that each induced response has its own temporal dynamics. Overall, it was expected that structural responses, such as leaf size and trichome induction are slower than chemical responses, such as glucosinolates and volatile induction. The temporal dynamics of the induced responses are also expected to be reflected in the dynamics of the expression profiles of the associated genes. Timing of induced responses to generalist, specialist or double herbivory may be dissimilar and therefore have a different effect on later arriving species. These differences in temporal dynamics may play a crucial role in determining the level of resistance or susceptibility to generalist and specialist herbivores.

In the present study, the temporal dynamics of various induced systemic responses in *B. juncea*, due to herbivory by the generalist insect of *Spodoptera* spp. (Chapter 2), the specialist insect *P. xylostella* or both these herbivores together (Chapter 3) were examined. Morphological (leaf size and trichome densities) and chemical (glucosinolates, amino acids, sugars) responses were analysed following damage over a period of 4 to 20 days. These two studies showed that temporal changes in structural and chemical traits are specific to the time after damage and the damaging herbivore. The induction of glucosinolates was slightly faster than that of trichomes when the plant was damaged by the generalist. On the contrary, trichomes were induced faster than glucosinolates when the specialist damaged the plant, and both defence traits reached the highest levels at the same time in case of double herbivory. This study therefore contradicts the hypothesis that chemical responses are faster than morphological responses. All other traits examined in these studies showed no significant response to any kind of herbivory, except leaf size, which increased in response to damage by the specialist, *P. xylostella* only.

The effect of these direct responses was assessed by studying the preference and performance of the larvae of both herbivore species. In all treatments, the generalist *S. litura* always preferred, and essentially performed better on,

undamaged plants as compared to damaged plants at all the time points tested. The specialist *P. xylostella*, on the other hand, always preferred the damaged plants. The degree of preference and performance, however, varied with time elapsed after the plant was damaged for both the herbivores. These results demonstrate the sensitivity of the generalist *S. litura* to induced responses in *B. juncea*, which may be exploited in pest management strategies for this crop in agricultural settings.

To investigate the influence of indirect defence responses in *B. juncea*, the temporal dynamics of herbivore-induced volatiles were analysed after herbivory by the generalist *S. exigua* (Chapter 4). The study was conducted over three days during which the plants were damaged. Multivariate analyses revealed that the volatile blend differed quantitatively as well as qualitatively on all three days. The expression analysis of 14 genes belonging to various hormonal, glucosinolate and volatile biosynthesis pathways showed that the temporal patterns of various volatile classes were reflected in their gene expression profiles. Moreover, the results clearly showed that herbivory by *S. exigua* induced the jasmonic acid pathway in *B. juncea*, whereas the salicylic acid pathway is slightly downregulated. Adult orientation bioassays determined that the generalist *S. litura* preferred undamaged plants to damaged plants. The specialist *P. xylostella*, and its parasitoid *Cotesia plutellae* preferred plants damaged for 72 h, which emitted isothiocyanates and sulfides in the highest proportions. The generalist parasitoid *C. marginiventris*, which also parasitizes *S. litura*, preferred plants damaged for 48 h, which may be related to significant changes in the emission of several general plant compounds, such as green leaf volatiles and terpenes. These studies thus indicate that specific compounds in the background of the whole volatiles blend may serve as a reliable cue for herbivores and their parasitoids, and that the temporal dynamics of the emitted plant volatile blends plays a key role in regulating these interactions.

Extrafloral nectar (EFN) in plants provides a carbohydrate-rich source of food for the natural enemies, thus acting as yet another indirect resistance mechanism

to herbivores. Although extrafloral nectaries are common among plants and are found in more than 114 plant families, they have never been reported in Brassicaceae. In Chapter 5, the occurrence of EFN in *B. juncea* is reported for the first time. It is secreted from the stem, flowering stalk and leaf axils only during flowering until silique formation, when the plant needs to be highly protected. The extrafloral nectaries are simple and cannot be seen with the naked eye. However, microscopic observations revealed a pocket-like structure, which was different from the structure of the floral nectaries of this plant. EFN was mainly composed of sugars, but also traces of amino acids and glucosinolates were found. Sugars and amino acids may provide essential nutrition to natural enemies, whereas glucosinolates may resist the entry of phytopathogens into the plant through the opening of extrafloral nectary. EFN secretion increased when the plant was damaged by above-and belowground chewing herbivores and aphids. Moreover, EFN production influences the arrestment of nectar-foraging parasitoids and is capable of sustaining them. Hence, EFN in *B. juncea* may act as a nutritious resource for parasitoids, and may thus act as an indirect resistance trait in *B. juncea*.

This thesis shows that herbivore induced responses in *B. juncea* are specific and dynamic in time at the physiological, behavioural as well as the gene expression level. Precise timing of induced responses may prove to be critical in designing novel strategies for plant resistance in agriculture. Moreover, the dynamic nature of induced responses, as shown by the studies in this thesis, may also explain seemingly opposite results of earlier studies that use the same plant-insect model systems (Chapter 6). Therefore, before considering induced responses as resistance factors, their temporal effect on generalist and specialist herbivores should be assessed. Hence, an understanding of temporal and spatial variations and system-wide assessment of induced responses in plants will help us identify the crucial mechanisms governing natural herbivore resistance in *B. juncea*.

De interacties tussen insecten en planten zijn zeer dynamisch. Om zichzelf te beschermen, produceren planten diverse structuren en chemische stoffen die het gedrag, de groei en/of het voortbestaan van herbivoren beïnvloeden. Sommige van deze stoffen en structuren zijn voortdurend aanwezig, ook als de plant niet wordt bedreigd. Deze op de achtergrond aanwezige afweer wordt constitutieve afweer genoemd. Maar na een aanval van insecten of pathogenen kunnen planten het gehalte bestaande afweerstoffen verhogen en/of beginnen met de productie van nieuwe structuren en chemische stoffen. Dit staat bekend als een geïnduceerde respons.

Geïnduceerde responsen zijn meestal slechts gedurende een bepaalde tijd op hun hoogtepunt en elke afzonderlijke respons heeft zijn eigen temporele dynamiek. De timing en plaats van elke respons hangen nauw samen met de belangrijkste functie van de respons. De responsen voor wondgenezing vinden bijvoorbeeld snel plaats. Daarentegen moeten voor sommige chemische stoffen biosynthetische routes worden geactiveerd, waarbij hormoonsignalering, genexpressie en eiwitsynthese moeten worden geactiveerd voordat deze stoffen kunnen worden geproduceerd. Als gevolg daarvan duurt het enige tijd voordat het geïnduceerde fenotype is gevormd en effect heeft op herbivoren en hun natuurlijke vijanden. Het vaststellen van het tijdsinterval tussen de verschillende veranderingen die in de plant optreden en hun samenhang en functie in tijd is daarom van fundamenteel belang om inzicht te krijgen in de rol die elke geïnduceerde respons speelt bij directe of indirecte afweer.

Het onderzoek in dit proefschrift is hoofdzakelijk gericht op het belang van de temporele dynamiek van verschillende directe en indirecte geïnduceerde responsen op moleculair, fysiologisch en ecologisch niveau in *Brassica juncea* (Indische/bruine mosterd, Brassicaceae). Voor deze onderzoeken werd *B. juncea* gekozen als modelsoort, omdat deze soort behoort tot de familie Brassicaceae, waartoe naast economisch belangrijke gewassen ook *Arabidopsis* behoort, die een modelplant is voor moleculaire, fysiologische en genetische onderzoeken. Daarnaast is er veel onderzoek gedaan naar de interacties van deze planten met

generalistische en specialistische herbivoren en de afweerstrategieën tegen deze herbivoren. Het onderzoek is voornamelijk gericht op: (a) het nauwkeurig beschrijven van de timing van geïnduceerde responsen op morfologisch, biochemisch en moleculair niveau in *B. juncea* als gevolg van door insecten aangerichte schade; (b) de ecologische effecten van directe en indirecte geïnduceerde afweer op verschillende momenten door middel van het bestuderen van het gedrag van generalistische en specialistische insecten. De belangrijkste hypothese van dit proefschrift is dat elke geïnduceerde respons zijn eigen temporele dynamiek heeft. In het algemeen werd verwacht dat structurele responsen, zoals bladafmetingen en trichoominductie, trager zijn dan chemische responsen, zoals glucosinolaten en de inductie van vluchtige stoffen. Er wordt ook verwacht dat de temporele dynamiek van de geïnduceerde responsen tot uitdrukking komt in de dynamiek van de expressieprofielen van de betrokken genen. De timing van geïnduceerde responsen op vraat door generalistische herbivoren, specialistische herbivoren of herbivoren van beide soorten, kan verschillend zijn en dus een ander effect hebben op soorten die later komen. Deze verschillen in temporele dynamiek kunnen een cruciale rol spelen bij het vaststellen van het afweer- of gevoeligheidsniveau ten opzichte van generalistische en specialistische herbivoren.

In onderhavig onderzoek werd de temporele dynamiek bestudeerd van verschillende geïnduceerde systemische responsen in *B. juncea* als gevolg van vraat door het generalistische insect *Spodoptera* spp. (hoofdstuk 2), door het specialistische insect *P. xylostella* of door beide herbivoren samen (hoofdstuk 3). Morfologische responsen (bladafmetingen en trichoomdichtheid) en chemische responsen (glucosinolaten, aminozuren, suikers) werden geanalyseerd gedurende een periode van 4 tot 20 dagen na het ontstaan van schade. Deze twee onderzoeken toonden aan dat temporele veranderingen in structurele en chemische eigenschappen kenmerkend zijn voor de tijd na het ontstaan van schade en de herbivoor die de schade veroorzaakt. Bij beschadiging van de plant door de generalist was de inductie van glucosinolaten enigszins sneller dan die van

trichomen. Bij beschadiging van de plant door de specialist werden er daarentegen sneller trichomen geïnduceerd dan glucosinolaten en bij vraat door beide herbivoren bereikten beide afweerstrategieën tegelijkertijd hun hoogste niveaus. Dit onderzoek weerlegt daarom de hypothese dat chemische responsen sneller zijn dan morfologische responsen. Bij bestudering van alle andere eigenschappen in deze onderzoeken werd geen significante respons aangetoond op welke vorm van vraat dan ook, met uitzondering van bladafmetingen, die alleen toenamen als respons op schade veroorzaakt door de specialist *P. xylostella*.

Het effect van deze directe responsen werd vastgesteld door de voorkeuren en prestaties van de larven van beide soorten herbivoren te bestuderen. Bij alle behandelingen verkoos de generalist *S. litura* op alle meetmomenten steeds onbeschadigde planten boven beschadigde planten en presteerde hij op alle meetmomenten in wezen beter op onbeschadigde planten dan op beschadigde planten. De specialist *P. xylostella* had daarentegen steeds een voorkeur voor de beschadigde planten. Bij beide herbivoren varieerde de mate van voorkeur en prestaties echter naarmate de tijd na beschadiging van de plant verstreek. Uit deze resultaten blijkt de gevoeligheid van de generalist *S. litura* voor de geïnduceerde responsen in *B. juncea*, die in de landbouw zouden kunnen worden gebruikt bij plaagbestrijding voor dit gewas.

Om de invloed van indirecte afweerresponsen in *B. juncea* te onderzoeken, werd er een analyse gemaakt van de temporele dynamiek van herbivoorgeïnduceerde vluchtige stoffen na vraat door de generalist *S. exigua* (hoofdstuk 4). Het onderzoek werd uitgevoerd gedurende een periode van drie dagen waarin de planten werden beschadigd. Uit multivariabele analyses bleek dat het vluchtige mengsel zowel kwantitatief als kwalitatief op alle drie de dagen anders was. De expressieanalyse van 14 genen die tot verschillende biosynthetische routes van hormonen, glucosinolaten en vluchtige stoffen behoren, toonde aan dat de temporele patronen van verschillende klassen vluchtige stoffen tot uitdrukking kwamen in hun genexpressieprofielen. Daarnaast toonden de resultaten duidelijk aan dat vraat door *S. exigua* de

jasmonzuurroute induceerde in *B. juncea*, terwijl de salicylzuurroute enigszins is gedownreguleerd. Door bioassays met volwassen exemplaren werd vastgesteld dat de generalist *S. litura* onbeschadigde planten boven beschadigde planten verkoos. De specialist *P. xylostella* en zijn parasitoïde *Cotesia plutellae* hadden een voorkeur voor planten die 72 uur eerder waren beschadigd. Deze planten gaven isothiocyanaten en sulfiden af in de hoogste verhoudingen. De generalistische parasitoïde *C. marginiventris*, die ook parasiteert op *S. litura*, had een voorkeur voor planten die 48 uur eerder waren beschadigd. Dit zou verband kunnen houden met de significante veranderingen in de afgifte van een aantal algemene plantenstoffen, zoals groene geurstoffen (GLV's) en terpenen. Deze onderzoeken duiden er dus op dat specifieke stoffen die in het gehele vluchtige mengsel op de achtergrond aanwezig zijn, zouden kunnen dienen als betrouwbaar signaal voor herbivoren en hun parasitoïden, en dat de temporele dynamiek van de door de plant afgegeven vluchtige mengsels een belangrijke rol speelt in het reguleren van deze interacties.

De extraflorale nectar (EFN) in planten vormt een koolhydraatrijke voedselbron voor natuurlijke vijanden en werkt dus ook als een indirect afweermechanisme tegen herbivoren. Ondanks het feit dat extraflorale nectariën veel voorkomen bij planten en in meer dan 114 plantenfamilies zijn aangetroffen, is er nooit melding van gemaakt in Brassicaceae. In hoofdstuk 5 wordt voor de eerste keer melding gemaakt van de aanwezigheid van EFN in *B. juncea*. Deze nectar wordt afgescheiden uit de stengel, de bloemsteel en de bladoksels, maar alleen tijdens de bloei tot de hauwvorming, als de plant veel bescherming nodig heeft. De extraflorale nectariën zijn eenvoudig en kunnen niet met het blote oog worden waargenomen. Bij microscopische observaties werd echter een zakachtige structuur aangetroffen, die afweek van de structuur van de florale nectariën van deze plant. De EFN bestond voornamelijk uit suikers, maar er werden ook sporen van aminozuren en glucosinolaten aangetroffen. Suikers en aminozuren kunnen een essentiële voedingsbron vormen voor natuurlijke vijanden, terwijl glucosinolaten bescherming kunnen bieden tegen het

binnendringen van fytopathogenen in de plant door de opening van extraflorale nectariën. De afscheiding van EFN nam toe als de plant werd beschadigd door boven- en ondergrondse kauwende herbivoren en bladluizen. De productie van EFN beïnvloedt daarnaast het aantrekken van op nectar foeragerende parasitoïden en kan deze parasitoïden in stand houden. De EFN in *B. juncea* kan daarom fungeren als een voedingsbron voor parasitoïden en kan dus in *B. juncea* werken als een indirecte afweerstrategie.

Dit proefschrift toont aan dat herbivoorgeïnduceerde responsen in *B. juncea* specifiek en dynamisch in tijd zijn op fysiologisch en gedragsmatig niveau en tevens op het niveau van genexpressie. Exacte timing van geïnduceerde responsen zou essentieel kunnen zijn bij het ontwikkelen van nieuwe strategieën voor plantafweer in de landbouw. De dynamische aard van geïnduceerde responsen, zoals aangetoond door de onderzoeken in dit proefschrift, kan tevens een verklaring zijn voor de ogenschijnlijk tegenstrijdige uitkomsten van eerdere onderzoeken waarbij dezelfde plant-insectmodelsystemen zijn gebruikt (hoofdstuk 6). Voordat geïnduceerde responsen kunnen worden beschouwd als afweerfactoren, moet daarom eerst hun temporele effect op generalistische en specialistische herbivoren worden vastgesteld. Inzicht in temporele en ruimtelijke variaties en het systeembreed vaststellen van geïnduceerde responsen in planten zullen ons dus helpen bij het identificeren van de cruciale mechanismen die verantwoordelijk zijn voor de natuurlijke afweer in *B. juncea* tegen herbivoren.

"Never lose an opportunity of urging a practical beginning, however small, for it is wonderful how often in such matters the mustard-seed germinates and roots itself "-Florence Nightingale

These words more or less summarize the beginning of my journey to this unimaginable PhD opportunity that I, a married woman and a mother of a child, originating from a developing country, received. The success of such a venture seemed impossible to everyone who heard of it, sometimes including myself, had it not been for the help, support, and encouragement from several people. The list is endless, and please excuse me if I may have missed someone unintentionally.

I will always remember the life-changing mail from Nicole ma'm in an answer to my mail with some practical questions about induced responses. You instantly took upon yourself to mentor me, giving not only my ideas and work, but also my whole life a positive direction. And thus began our association of already 8 years, much before I started my PhD. Your firm belief that a better way can always be found, has influenced my approach to this research, as well as to my daily life. Your clear intellect and an unstinting belief in me has always been a source of inspiration for me to go on. I owe my heartiest gratitude to you for being not only the world's best supervisor and the superwoman that you are, but also for being an understanding and true friend.

Words fail me in expressing my gratitude to Reddy sir, who has been my guru, boss and a guardian angel, before you agreed to be my co-promoter. You guided me with your outstanding administrative abilities during the toughest periods of my PhD and came out with exactly the right solutions time and again. Your incredible mentoring, energy, and positive outlook are an inspiration to many, including myself.

I am deeply indebted to Louise Vet ma'm for not only accepting me as your PhD student without even knowing me, but also for always finding time for me despite your round-the-clock schedule. Your comments on each chapter draft are themselves a course of critical thought upon which I will always draw. Thank you very much ma'm for being instrumental in turning my dreams and ambitions to reality.

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I am grateful to my project attendant Om Prakash Singh, whose dedication went far beyond the call of duty. You have been the friend and colleague with whom I could share all my anticipations and fears during the project without worries of being judged.

My first stay in NL gave me friends for lifetime: Paulina, Katherine, Katrin, Taia, Baoli and Maja. You people ensured that I never got homesick. Paulina and Katrin, thanks a lot for letting me stay in your house when I had courses in Wageningen. I always felt at home talking and laughing with your family. Many thanks to my dear friend Ciska, whose skills and experience are highly valued. Every time we worked together while sharing jokes and chatting, I was always amazed at the ease at which things could be done. I also thank you for accepting to be my paranymph and making all the arrangements so efficiently.

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My stay in Rennes was short, but gave me memories for life. For that, I owe a big thanks to Prof. Anne-Marie Cortesero for not only accepting me in your lab for those four months, but also for your excellent guidance, encouragement and support throughout my stay. Your sunshine smile each morning, mixed with advices and jokes were an instant energy booster for the whole day. The stay in Rennes wouldn't have been possible but for the unstinting personal and professional support by Prisca and André in whom I found a family during my stay there. I am grateful for the timely help given to me by Valerie, Sonia, Crystelle and Veronique that made my experiments much easier than they seemed when I designed them.

The final year of PhD is normally considered to be the toughest. But I never felt the heat of it, thanks to my dear friend, philosopher and guide, Tom. You were very patient while clearing my concepts and answering even my silly queries. Special thanks for giving me a ride in your car endless times from Wageningen to Nijmegen and for accepting to be my paranymph. I will always treasure all the moments of brainstorming, fun and fights that we shared. Thanks a lot Onno, Holger, Duy, Nadia and Rob for all the good times in RU. I would not have managed to accommodate in that new environment so easily if you people were not there with all that good humor and witty chats. EGG rocks!

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came to the staff room of Zoology for my daily dose of fun and enjoyment, while in Delhi. Thanks a lot…I am proud to be a part of the 'Zoo'logy gang.

I cannot complete my acknowledgements without expressing my deep gratitude to my family. Mamma, Daddy and Beena Bua, I am eternally grateful to you for your unconditional support and encouragement in letting me pursue my interests, even when the interests went beyond boundaries of language, field and geography. You took the best care of Aarav, and never let him feel my absence. Without you, I wouldn't have dared to leave my son and go so far for such a long time to pursue my own dreams.

To have "Dr." in front of my name has been a dream of my mom and dad even before I could spell my own name. You both never gave up on me and always encouraged me to go on, giving me that necessary nudge whenever I felt disheartened during my study period. Thanks for being there, always. Thank you very much Varun and Swati for your constant encouragement, prayers and wishes. My special thanks to dearest Vaanya for always bringing a smile on my face.

I am especially indebted to my better half, Amit, whose loving care and belief in me has sustained me during my PhD period. You have always been a great friend and support with whom I can share not only all my happiness, but also all my fears, apprehensions and sorrows. You are a gem of a person and I have yet to come across a better human being than you. Thank you for being part of my life.

Finally, I dedicate my PhD to the person who has sacrificed the most during this period…my darling son, Aarav. I never took your permission for leaving you and going away to NL for months together, since you were only two years old. But you never made me feel guilty for it even when you grew up enough to complain, and in fact you defended me time and again when people snubbed me for it. The day I submitted my thesis, you danced around the whole house, and wrote a touching note to me. It was then that I realized you have been living my dream too all these years and how integrally you are attached to me. I will always be indebted to you for giving me a chance to pursue my dreams.

Warm regards
Vartika

Vartika Mathur was born on the 27th of August 1979 in New Delhi, India. After finishing her secondary education, she pursued her Bachelors in Zoology (Hons.) (1997-2000) and Masters in Zoology (2000-2002) from the University of Delhi with specialization in entomology. In 2003, under the supervision of Prof. dr. Ashok K. Singh (Department of Zoology, University of Delhi), Vartika was awarded an M. Phil. for her work on the biology and life tables of *Spilosoma obliqua*. In the latter half of 2003, she cleared the National Eligibility Test (NET) for teaching and started teaching Zoology to undergraduate students of University of Delhi. From February to July 2006, she worked as a research fellow on the inhibitory studies of Trifluoperazine (TFP), a calmodulin antagonist in Mycobacteria. This study was conducted under the supervision of Dr. Hemalatha Reddy (Sri Venkateswara College, University of Delhi). In September 2006, she was appointed as an Assistant Professor in Sri Venkateswara College, University of Delhi, on permanent basis, a position she holds till date.

Vartika she wrote a proposal to Nuffic in 2007 to pursue PhD on the induced responses of *Brassica juncea*. In May 2008, she started her PhD under the supervision of Prof. dr. Louise Vet and Prof. dr. Nicole van Dam at NIOO, Wageningen. Accordingly, she studied the temporal dynamics of herbivore induced morphological and chemical responses in *Brassica juncea* together with their effects on herbivores and their parasitoids. This work was carried out in Netherlands and India over a period of 4 years. In India, Vartika was awarded two grants by the University Grants Commission to pursue her research on the induced responses of *B. juncea.* With the first grant she worked as a co-investigator from 2008-2010 and with the second grant as the Principal investigator from 2011-2014. She was also given the European Mobility grant by the International Doctoral College of the European University of Brittany (CDI-UEB) to work from April to August 2010 on the effects of extrafloral nectar in *B. juncea* and their behavior and longevity, above and below ground parasitoids. This research was performed under the supervision of Prof. dr. Anne Marie Cortesero (Department of Insect Ecobiology, University of Rennes, France). The results of the aforementioned studies have been described in this thesis.

Published

- **Mathur, V.**, Wagenaar, R., Caissard, J. C., Reddy, A. S., Vet, L. E. M., Cortesero, A. M. and van Dam, N. M. (2012). A novel indirect defence in Brassicaceae: Structure and function of extrafloral nectaries in *Brassica juncea*. Plant, Cell and Environment (in press).
- **Mathur, V.**, Ganta, S., Raaijmakers, C. E., Reddy, A. S., Vet, L. E. M. & van Dam, N. M. (2011) Temporal dynamics of herbivore-induced responses in Brassica juncea and their effect on generalist and specialist herbivores. Entomologia Experimentalis *et* Applicata 139(3): 215-225.
- **Mathur, V.** (2010). Environmental Ecology and Field Biology: Applied aspects. IK Publishing House, New Delhi, India.
- Qiu, B., Liu, L., Li, X-Xi., **Mathur, V.**, Qin, Z-Q.andRen, S-X. (2009). Genetic mutations associated with chemical resistance in the cytochrome P450 genes of invasive and native *Bemisiatabaci* (Hemiptera: Aleyrodidae) populations in China. Insect Science 16: 237-245.
- Qiu, B., Chen, Y.P., Liu, L., Peng, W.L., Li, X.X., Ahmed, M.Z., **Mathur, V.**, Du, Y.Z. and Ren, S.X. (2009). Identification of three major *Bemisiatabaci* biotypes in China based on morphological and DNA polymorphisms. Progress in Natural Science 19: 713-718.

Submitted

- **Mathur, V.**, Tytgat, T. O. G., de Graaf, R. M., Kalia, V., Reddy, A. S., Vet, L. E. M. and van Dam, N. M. Dealing with double trouble: consequences of single and double herbivory in *Brassica juncea*.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Vartika Mathur
Date: 18 September 2012
Group: NIOO-KNAW and Entomology, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Temporal dynamics of molecular, chemical and morphological induced responses in Indian mustard (Brassica juncea)	Sep 04, 2008
► **Writing or rewriting a project proposal**	
Temporal dynamics of molecular, chemical and morphological induced responses in Indian mustard (*Brassica juncea*)	Aug 28, 2008
► **Writing a review or book chapter**	
► **MSc courses**	
Genetics and ecogenomics (RU, NL)	Sept 12-23, 2011
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
► **EPS theme symposia**	
EPS symposium :ecology and enviromental plant Sciences 2" (Wageningen)	Sep 22, 2009
EPS theme 2 symposium/ Willie Commelin Scholten Day: (Amsterdam)	Feb 03, 2011
EPS theme 2 symposium (Wageningen)	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
NWO lunteren day	Sep 17, 2008
Netherlands Annual Ecology Meeting, Luntern	Feb 09, 2011
ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	April 03, 2012
► **Seminars (series), workshops and symposia**	
Seminar: "Indirect defences in facultative and obligate plant-carnivore mutualism- field study in Mexico"	May 21, 2008
► **Seminar plus**	
► **International symposia and congresses**	
BES annual symposium on "Integrative role of plant secondary metabolites in ecological systems" (Sussex, UK)	Apr 12-14, 2010
ISCE annual meeting (Tours, France)	Jul 31-Aug 04, 2010
Symposium Insect Plant Relationship (SIP) 14 (Wageningen)	Aug 13-18, 2011
► **Presentations**	
Presented a poster in Utrecht summerschool	Aug 24-26, 2009
Presented a poster in BES annual symposium (Sussex, UK)	Apr 12-14, 2010
Gave an oral presentation in ISCE annual meeting (Tours, France)	Jul 31-Aug 04, 2010
Gave an oral presentation in EPS theme 2 synposium/Willie Commelin Scholten Day	Feb 03, 2011
Presented a poster in SIP14 (Wageningen, NL)	Aug 13-18, 2011
Gave an oral presentation at the ALW meeting 'Experimental Plant Sciences' (Lunteren, The Netherlands)	April 03, 2012
► **IAB interview**	Feb 18, 2011
► **Excursions**	
Subtotal Scientific Exposure	*12.5 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPS summer school on Environmental Signalling (Utrecht, NL)	Aug 24-26, 2009
PhD course 'Bioinformatics - a user's approach'	Aug 29-Sept 02, 2011
► **Journal club**	
► **Individual research training**	
Worked in the laboratory of Parasitoid Ecobiology at the University of Rennes, France	Apr 15- Aug 08, 2010
Subtotal In-Depth Studies	*5.4 credits**

4) Personal development	date
► **Skill training courses**	
WGS Course: Information Literacy, including Introduction Endnote	May 27 & 28, 2008
Basic statistics course	Jun 16-20, 2008
PhD competence assessment	Sep 18, 2008
Techniques for Writing and Presenting a Scientific Paper	Sep 01-04, 2009
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32**

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

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

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