Multitrophic effects of plant resistance:

from basic ecology to application in transgenic crops

Martine Kos

Thesis committee

Thesis supervisors
Prof. dr. M. Dicke
Professor of Entomology
Wageningen University

Prof. dr. L.E.M. Vet
Professor of Evolutionary Ecology
Wageningen University

Thesis co-supervisor
Dr. ir. J.J.A. van Loon
Associate Professor at the Laboratory of Entomology
Wageningen University

Other members:
Prof. dr. ir. W.H. van der Putten, Wageningen University
Prof. dr. N.M. van Straalen, VU University Amsterdam
Dr. T.J. de Jong, Leiden University
Prof. dr. ir. N.M. van Dam, Radboud University Nijmegen

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

Multitrophic effects of plant resistance:

from basic ecology to application in transgenic crops

Martine Kos

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 16 March 2012
at 4 p.m. in the Aula.

Martine Kos
Multitrophic effects of plant resistance: from basic ecology to application in transgenic crops,
303 pages.

Thesis, Wageningen University, Wageningen, NL (2012)
With references, with summaries in Dutch and English

ISBN 978-94-6173-206-4

An understanding of the natural world and what's in it is a source of not only a great curiosity but great fulfilment.

- David Attenborough

Abstract

Plants have evolved a wide array of direct and indirect resistance traits that prevent or reduce herbivory by insects. The aim of this thesis was to study the effects of direct and indirect plant resistance traits on the multitrophic interactions between brassicaceous plants, leaf-chewing and phloem-sucking aboveground herbivores and their natural enemies, parasitoids and predators. *Brassica oleracea* cultivars and *Arabidopsis thaliana* ecotypes were used that differ in production of glucosinolates or emission of volatiles, secondary plant chemicals acting in direct and indirect resistance respectively. There was a considerable intraspecific variation in the multitrophic effects of plant resistance traits in both plant species. In the field, bottom-up forces (plant chemistry and morphology) appeared more important for herbivore abundance than plant-mediated top-down forces (attraction and arrestment of natural enemies). Under greenhouse conditions, glucosinolates affected the performance of herbivores and that of their natural enemies. The performance of both a generalist and a specialist caterpillar was negatively correlated with glucosinolates in the plant, whereas that of a parasitoid of the specialist caterpillar was positively correlated with glucosinolates. Performance of a specialist aphid was positively correlated with phloem glucosinolates, and the aphid selectively sequestered these glucosinolates. Glucosinolates and their volatile hydrolytic products correlated negatively with the performance and behaviour of one of the predators of this aphid, but positively with that of one of its parasitoids. These results suggest that direct and indirect resistance traits can be in conflict, but they can also work in concert to enhance resistance to herbivores, depending on the biology of the herbivore and carnivore involved. Transgenic *A. thaliana* plants engineered to emit larger amounts of volatile terpenoids repelled the aphid, attracted the parasitoid, but did not affect predator behaviour.

These fundamental ecological results provided the background information required to strengthen ecological risk analysis for transgenic plants in the framework of the programme 'Ecology Regarding Genetically modified Organisms' funded by the Dutch government. The effects of transgenic plants on non-target organisms were compared with the baseline variation in the effects on non-target organisms that exists among conventional varieties or, in the case of *A. thaliana*, wild ecotypes. Four *B. oleracea* cultivars and three *A. thaliana* ecotypes were selected to represent the baseline variation. The

baseline variation in effects on target and non-target organisms was relatively consistent over different environments, soil types and time. The effects of transgenic *A. thaliana* plants altered in direct and indirect resistance on non-target organisms were mostly within the baseline variation in these effects. Finally, the knowledge gained was applied to develop guidelines for governmental regulators that can be used to assess the potential ecological effects of transgenic crops on non-target organisms, in relation to baseline variation.

Contents

1

General Introduction

Martine Kos

"I am tempted to give one more instance showing how plants and animals, remote in the scale of nature, are bound together by a web of complex relations."

Charles Darwin - On the origin of species by means of natural selection (1859)

1.1 Multitrophic interactions

Green plants make up the largest part of all biomass in terrestrial ecosystems. Insects have the most species of any class of organisms on our planet, and about half of the known insect species are herbivorous. The interactions between plants and insects are therefore among the most common interactions in ecosystems, and plants and insects have been evolving together for over one hundred million years (Schoonhoven et al. 2005). However, the interactions between plants and insects cannot be fully understood without considering the third trophic level: the natural enemies of herbivores (Price et al. 1980). Carnivorous natural enemies can have large effects on the structure and dynamics of herbivore communities via predator-prey interactions. They may also, indirectly, influence the abundance of plant species (Schmitz et al. 2000; Halaj and Wise 2001). In reverse, effects of plants on herbivores can 'cascade up' the food web and determine species diversity and population dynamics at higher trophic levels (Hunter and Price 1992; Bukovinszky et al. 2008). Although often studied separately, insect communities are structured by complex interactions between these bottom-up (resource-based) and top-down (natural enemy-based) forces (Hunter and Price 1992; Forkner and Hunter 2000; Dicke and Hilker 2003; Aquilino et al. 2005).

Plants play an essential role in mediating the interactions between a multitude of organisms. Their traits can affect not only the herbivores that feed on the plant, but also the natural enemies of these herbivores, and even organisms at the fourth trophic level (Hunter and Price 1992; Harvey et al. 2003; Soler et al. 2005; Bukovinszky et al. 2008). Plants can also mediate the interactions between insects at higher trophic levels, such as the larvae of different parasitoid species (Poelman et al. 2011). Furthermore, plants function as essential links between aboveground and belowground organisms (van der Putten et al. 2001; Wardle et al. 2004b; Bezemer et al. 2005; Bezemer and van Dam 2005). For example, damage by belowground herbivores may change the chemistry of the plant and, thereby, influence the performance and

behaviour of aboveground carnivores (Soler et al. 2005; Soler et al. 2007).

A wide array of direct and indirect plant resistance traits that prevent or reduce herbivory by insects has evolved. These traits can affect the performance or behaviour of herbivores directly by chemical means, such as the production of toxins, repellents and digestibility reducers, or by physical means, such as the production of trichomes and epicuticular waxes (Karban and Baldwin 1997; Schoonhoven et al. 2005). Plants may maintain a large variation in the production of chemicals to confer resistance to many different attackers (Jones and Firn 1991; Newton et al. 2009b; Poelman et al. 2009b), and might not suffer from trade-offs between the different chemical resistance traits (Koricheva et al. 2004).

Plants can also affect herbivores indirectly, by promoting the effectiveness of natural enemies that feed on these herbivores. For example, plants can provide natural enemies with refuges or alternative food sources such as extrafloral nectar (Heil 2008; Kessler and Heil 2011). Plants can also emit herbivore-induced volatile organic compounds (VOCs) that attract natural enemies of herbivores to the plant (Turlings et al. 1990; Vet and Dicke 1992; Takabayashi et al. 2006; Dicke and Baldwin 2010; Shiojiri et al. 2010; Hare 2011; Kessler and Heil 2011). VOCs can be reliable cues indicating the presence of suitable hosts or prey for the natural enemies of herbivores (Vet and Dicke 1992; Dicke 1999a; Takabayashi et al. 2006). In some cases, natural enemies have an innate preference for the VOC blend induced by their host or prey, whereas in other cases natural enemies have to learn the association between the VOC and the presence of the host or prey (reviewed in Takabayashi et al. 2006). VOCs also play roles in interactions with other community members such as herbivores, pathogens, pollinators, and neighbouring plants (Dicke and van Loon 2000; Bruinsma and Dicke 2008; Dicke and Baldwin 2010; Kessler and Heil 2011).

Direct and indirect resistance mechanisms can be constitutive, *i.e.* expressed independently of the presence of an attacker, or they can be induced upon feeding or oviposition by herbivores (Karban and Baldwin 1997; Dicke and Hilker 2003; Schoonhoven et al. 2005; Hilker and Meiners 2011; Karban 2011). Induced resistance can be beneficial for the plant by saving costs when attackers are absent, and it allows the plant to respond specifically to certain attackers (Karban and Baldwin 1997; Dicke and Hilker 2003; Schoonhoven et al. 2005).

There is variation in plant resistance traits, not only between plant species, but also within plant species. It has been shown that intraspecific variation in plant resistance traits can have large effects on insect communities (Crutsinger et al. 2006; Johnson et al. 2006; Newton et al. 2009b; Poelman et al. 2009b). For example, differences in concentrations of secondary metabolites among wild plant populations or cultivars can affect the abundance of insect herbivores, and consequently the abundance of their natural enemies (Whitham et al. 2003; Bukovinszky et al. 2008; Newton et al. 2009b; Poelman et al. 2009b). Furthermore, intraspecific differences in quality or quantity of plant volatile blends can result in differential attraction of natural enemies of herbivores (Agrawal et al. 2002; Hoballah et al. 2002; Rasmann et al. 2005; D'Alessandro et al. 2006; Gols et al. 2009; Poelman et al. 2009a).

With some exceptions (see Sznajder and Harvey 2003; Reudler Talsma 2007; Gols and Harvey 2009 and references therein), direct and indirect resistance strategies are mostly studied independently, disregarding the potential evolutionary conflict between them (Fig. 1.1). For example, secondary plant metabolites that confer direct resistance to herbivores can also negatively influence the performance of the natural enemies of these herbivores, either directly due to toxicity of the compounds, or indirectly due to reduced growth and development of their host or prey (Harvey 2005; Soler et al. 2005; Ode 2006; Gols and Harvey 2009). On the other hand, volatile breakdown products of secondary plant metabolites can attract natural enemies of herbivores because they indicate the presence of their host or prey (Bradburne and Mithen 2000; Blande et al. 2007; Mumm et al. 2008), resulting in a dual defensive role of these secondary metabolites.

To understand the effects of direct and indirect plant resistance traits on the insect community, it is important to study the multitrophic effects of these traits. The aim of this thesis was to study the effects of direct and indirect plant resistance traits on the multitrophic interactions between plants, aboveground herbivores and their natural enemies. The following fundamental ecological research objectives were developed:

1) To study the effects of direct and indirect plant resistance traits on herbivores with different feeding strategies and/or levels of specialisation;
2) To study the effects of the same direct and indirect plant resistance traits on predators and parasitoids;

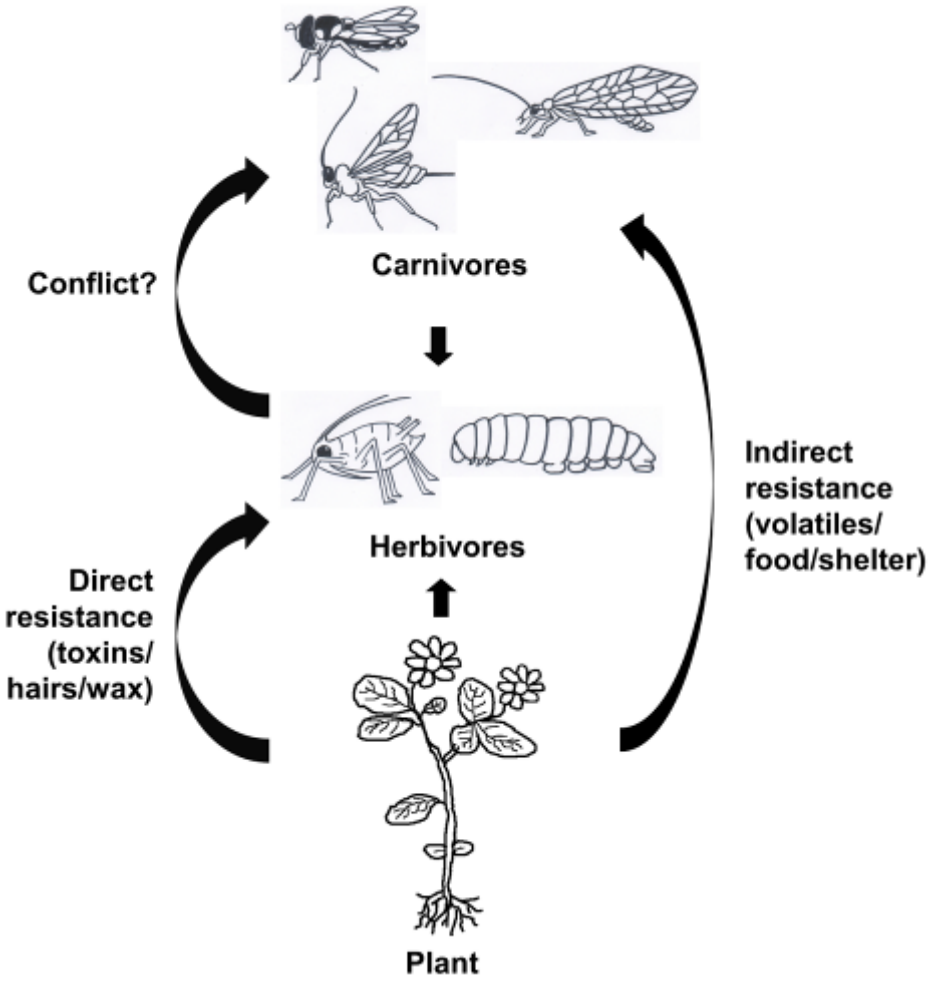


Fig. 1.1 *The multitrophic effects of plant resistance traits. Direct plant resistance traits, such as toxic secondary metabolites or hairs, affect the performance or behaviour of herbivores directly. Indirect resistance traits, such as the emission of herbivore-induced volatiles or the provision of alternative food or shelter, promote the effectiveness of natural enemies. Secondary plant metabolites that confer resistance to herbivores might also negatively affect the natural enemies of these herbivores, potentially leading to an evolutionary conflict between direct and indirect plant resistance. Insect drawings by Cindy ten Broeke.*

A multidisciplinary approach, combining both chemical (plant and insect chemistry) and ecological (performance, abundance and behaviour of insects aspects, was used to address these objectives under controlled greenhouse and laboratory conditions, as well as under natural field conditions.

As discussed, plants that possess natural resistance traits can affect the natural enemies of attacking herbivores as well. Similarly, plants that are genetically engineered for an enhanced direct or indirect resistance against pest herbivores may also exhibit traits that affect the natural enemies of these herbivores. Therefore, information on the multitrophic effects of plant resistance traits is essential in studying the ecology of such transgenic plants. In this thesis, the ecological effects of transgenic plants on natural enemies of herbivores are addressed, and the knowledge that is gained during the fundamental ecological studies is applied in this respect.

1.2 Transgenic crops

Transgenic plants are genetically engineered to contain DNA from an organism belonging to a different species (if the DNA is endogenous to the plant species, the plants are called cisgenic plants, but in this thesis the term 'transgenic' will be generically used for genetically modified plants). Transgenic plants can contain genes that confer resistance to insects, pathogens, herbicides or abiotic stresses, or genes that improve the nutritional value, storability or flavour (Nap et al. 2003). These traits can be attained much faster by genetic engineering than by conventional breeding programs. Farmers can gain benefits from growing insect-resistant transgenic crops, such as a reduction in production costs due to reduced pest management and labour, and yield increases due to reduced pest damage (Kalaitzandonakes 1999). Since their first commercialisation in the mid 1990s, the cultivation of transgenic crops has increased enormously, from 2 million hectares in 1996 to 148 million hectares in 2010 (James 2010). At present, all commercially available insect-resistant transgenic crops are aimed at direct resistance against pests, and all of these express genes coding for *Bacillus thuringiensis* (*Bt*) toxins that negatively affect specific herbivores (Aronson and Shai 2001; Chen et al. 2008). Transgenic crops with enhanced attraction of natural enemies of pest herbivores are not yet commercially available.

1.2.1 Ecological risks of growing transgenic crops

With the rapid global increase in the cultivation of transgenic crops and the many new biotechnological developments, a discussion on the urgency of obtaining insight in the ecological effects of transgenic crops arose. This ecological knowledge was considered essential in the assessment of the environmental risks of transgenic crops, but the acquisition of the data lagged

behind the biotechnological developments (Schuttelaar & Partners et al. 2004). Several ecological issues have to be considered before introducing transgenic crops into the environment (Conner et al. 2003; Dutton et al. 2003; Scholte and Dicke 2005; Snow et al. 2005; Romeis et al. 2006). The concerns relate to the invasion of transgenic crops into natural habitats, hybridization of the transgenic crops with wild relatives, horizontal gene transfer, development of resistance in target organisms and effects on non-target organisms (Conner et al. 2003; Snow et al. 2005). In this thesis, the target organism is defined as the organism to which the transgene is aimed to confer resistance, whereas the non-target organism is defined as any organism associated with the crop that is not the intended target of the transgenic crop. Of the ecological concerns, the potential negative effects on non-target organisms are of major importance (Groot and Dicke 2002; Romeis et al. 2008a). Effects of transgenic plants on non-target organisms can be due to the intended effects of the transgenic crop, *e.g.* as a result of the toxic effects of the *Bt*-toxin produced by *Bt*-crops (Groot and Dicke 2002; Clark et al. 2005). However, transgenic crops can also have effects caused by unintended changes in the metabolism and physiology of the transgenic plant (Dutton et al. 2003; Snow et al. 2005; Birch et al. 2007), such as lower glucosinolate levels in transgenic canola compared to conventional lines (Daun 2004), and higher lignin content in several commercial varieties of *Bt*-corn (Saxena and Stotzky 2001). Such unintended changes may only become clear under specific environmental conditions (Birch et al. 2007).

1.2.2 The ERGO-project

European Union (EU) regulatory bodies require detailed environmental risk assessments (ERAs) before they permit the introduction of a transgenic crop into the agro-ecosystem. Because in the EU transgenic plants are seen as new entities, risk assessments for transgenic crops are different from standard risk assessments that apply to conventional plant protectants in that they require the evaluation of potential adverse effects of the introduction of the transgenic crops on the environment (European Community 2001). However, at the start of this thesis project in 2007, the EU did not provide clear guidelines with regard to how such ecological risk assessments should be carried out.

The Dutch government considered it problematic that there is a lack of ecological knowledge on transgenic crops and of clear guidelines for ERAs.

Therefore, in 2007, the Dutch government fostered a research programme aimed at strengthening the ecological risk analysis for transgenic plants: the ERGO (Ecology Regarding Genetically modified Organisms) programme. The main objective of the ERGO-programme is to study the ecology of transgenic crops to be able to develop ecology-based guidelines for assessing the ecological effects of new transgenic crops. The ERGO-programme is based on three desktop studies by the Dutch Advisory Commission on Genetic Modification (COGEM) (Groot et al. 2003; Knols and Dicke 2003; Kowalchuk et al. 2003), and encompasses three fields of special interest:

1) Multitrophic interactions in transgenic crops;
2) Effects of hybridisation and introgression between crops and wild relatives;
3) Effects of transgenic crops on the functioning of soil ecosystems.

One of the projects within the ERGO-programme links the first and the third field of interest and focusses on the ecological effects of transgenic crops on aboveground and belowground non-target organisms. The current thesis results from this project. The aim of the project is: “Development of an ecological method to evaluate the effects of transgenic crops, altered in direct and indirect plant resistance traits, on non-target organisms in relation to baseline information”. The baseline information refers to the variation in the effects on non-target organisms that already exists among conventional varieties of the crop species. The project involves three PhD-students. PhD-1 (this thesis) focusses on aboveground non-target organisms, PhD-2 focusses on belowground non-target organisms (Kabouw 2012), and PhD-3 focusses on the characterisation of direct and indirect plant resistance traits (Houshyani 2012).

In accordance with the general aim of the ERGO-programme, the applied research objectives of this thesis are:

1) To assess baseline variation in effects of direct and indirect plant resistance traits on aboveground non-target organisms;
2) To compare the baseline variation with effects of transgenic plants altered in direct and indirect resistance traits on non-target organisms to assess whether transgenic effects exceed baseline variation;
3) To assess the validity of using greenhouse experiments to predict non-target effects in the field;

4) To develop guidelines for regulators to assess the effects of transgenic crops on non-target organisms in relation to baseline information, a combined effort with the other two PhD-students (Houshyani 2012; Kabouw 2012).

The information on the interactions between plants and insects that will be gained during the fundamental ecological studies is considered essential to address these applied objectives.

1.2.3 The baseline variation

Conventionally bred varieties of a crop species display a certain range of variation in the effects on non-target organisms. This variation can potentially be larger than the variation between one transgenic crop and the original genotype into which the transgene was introduced (the isogenic line). To properly assess the ecological effects of a transgenic crop, it is necessary to represent the 'baseline variation': the variation in effects observed among a selection of non-transgenic varieties, across sets of environmental conditions. By comparing the effects of the transgenic crop with this baseline variation, it is possible to assess whether the transgenic plant is disproportionally affecting non-target organisms compared to the varieties that were produced by traditional breeding. For a proper representation of the baseline variation, fundamental ecological knowledge on the interactions between plants and insects in the study system is required.

1.3 The study system

1.3.1 Plant material

This thesis focusses on plants of the family Brassicaceae. This family contains important crops such as cabbage, broccoli, cauliflower, turnip, mustards and oilseed rape, as well as the model plant *Arabidopsis thaliana* (L.) Heynh. Brassicaceous plants, also known as crucifers, produce several compounds that are involved in direct and indirect plant resistance, such as glucosinolates, their volatile breakdown products and other VOCs (van Poecke and Dicke 2004; Bukovinszky et al. 2005; Hopkins et al. 2009; Shiojiri et al. 2010). Glucosinolates (GLS) are among the best-studied secondary plant metabolites. Upon tissue damage, the GLS that are stored in the vacuoles become exposed to the enzyme myrosinase, which is stored separately in special cells. As a result of the myrosinase activity, GLS are hydrolysed into several toxic compounds such as (iso)thiocyanates and nitriles. These

breakdown products negatively affect a wide variety of generalist herbivores (Bones and Rossiter 2006; Halkier and Gershenzon 2006; Hopkins et al. 2009). Specialist herbivores of Brassicaceae, however, have evolved specific adaptations to detoxify GLS or inhibit the formation of toxic (iso)thiocyanates (Ratzka et al. 2002; Wittstock et al. 2004), sequester GLS (Francis et al. 2001b; Kazana et al. 2007; Müller 2009), or use GLS and their hydrolysis products as oviposition or feeding stimulants (van Loon et al. 1992; Gabrys and Tjallingii 2002; Miles et al. 2005). GLS can also affect the natural enemies of herbivores (reviewed in Gols and Harvey 2009). For example, GLS from the plant can negatively affect the performance of parasitoids and predators that feed on herbivores of brassicaceous plants (Francis et al. 2001b; Vanhaelen et al. 2002; Sznajder and Harvey 2003; Soler et al. 2005). Furthermore, volatile breakdown products of GLS act as attractive compounds to several specialist parasitoids of herbivores that feed on GLS-containing plants (Bradburne and Mithen 2000; Blande et al. 2007; Mumm et al. 2008). Brassicaceous plants also emit other classes of VOCs that are involved in the attraction of natural enemies, such as terpenoids and green leaf volatiles (Mumm and Dicke 2010; Shiojiri et al. 2010).

The Brassicaceae plant family exhibits a large variation in direct and indirect resistance traits, providing an ideal system to address the fundamental and applied objectives of this thesis. In wild cabbage (*Brassica oleracea* L.), for instance, centuries of breeding have resulted in a large number of varieties, also called cultivars, that differ in the biosynthesis of GLS (Kushad et al. 1999; Gols et al. 2008b; Poelman et al. 2009b; Kabouw et al. 2010b) and the attraction of natural enemies (Chin and Lindsay 1993; Geervliet et al. 1997; Kalule and Wright 2004; Poelman et al. 2009a). The Laboratory of Entomology has a long history in studying Brassicaceae-insect interactions (see *e.g.* the theses of Geervliet 1997; Vos 2001; Broekgaarden 2008; Bruinsma 2008; Gols 2008; Poelman 2008; Yang 2008; Snoeren 2009). Based on the results of two previous PhD-theses (Broekgaarden 2008; Poelman 2008), four white cabbage cultivars (*B. oleracea* L. convar. *capitata* var. *alba*) that differ in their direct and indirect resistance to herbivores were selected. These four cultivars were used to represent the baseline variation in effects of resistance traits of cultivated plants on herbivores and their natural enemies.

Because it is easily transformed and has a short lifecycle (Aharoni et al. 2003), the model plant *A. thaliana* was selected for the genetic transformations. Different *A. thaliana* ecotypes have been shown to differ in

production of GLS and VOCs (Kliebenstein et al. 2001; Huang et al. 2010; Snoeren et al. 2010; Houshyani et al. 2012). Based on the results reported in the thesis of Houshyani (2012), three *A. thaliana* ecotypes were selected to represent the baseline variation in effects of resistance traits of non-crop plants on herbivores and their natural enemies.

This baseline variation could then be compared with the effects of genetically transformed *A. thaliana* lines with enhanced direct or indirect resistance against herbivores.

1.3.2 Selection of non-target species

For pragmatic reasons, this thesis focusses on one specific group of non-target organisms: the carnivorous arthropods (predators and parasitoids). Toxic effects of transgenic crops on these non-target organisms are a major concern because these carnivores are the natural enemies of (possible) pest species and play an important role in natural pest regulation (Groot and Dicke 2002; Dutton et al. 2003; Romeis et al. 2006; van Lenteren 2008; Gagic et al. 2011; van Lenteren 2012). Furthermore, natural enemies are likely to get exposed to the transgenic product if they feed on herbivores that fed on the transgenic plant. These are important criteria for selecting non-target organisms that should be included in risk assessments (Bruinsma et al. 2003; Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Andow and Zwahlen 2006; Birch et al. 2007; Prasifka et al. 2008; EFSA 2010).

Natural enemies belonging to different functional groups that differ in their feeding strategy, *i.e.* predators and parasitoids (Table 1.1), were selected, because these are expected to be differentially affected by (herbivore-mediated) plant resistance traits. Most predators kill their prey immediately and feed on multiple prey individuals during their development. Parasitoids, on the other hand, develop inside a single host individual and koinobiont parasitoids, which attack hosts that continue to feed and grow after parasitism, feed selectively on certain host tissues (Godfray 1994; Harvey 2005). Natural enemies of phloem-feeding herbivores (aphids), as well as natural enemies of leaf-chewing herbivores (caterpillars), were selected (Table 1.1), because these herbivores have different feeding strategies and are therefore expected to be differentially affected by plant resistance traits. Because this thesis focuses on brassicaceous plants, the selected non-target species and their host or prey items were chosen to be naturally associated with brassicaceous plants.

Table 1.1 Selected species of natural enemies and the host or prey species that was provided to them in the studies described in this thesis

	Natural enemy	Level of specialisation	Provided host or prey	Level of specialisation
Parasitoid (koinobiont)	*Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae)	Specialist	*Plutella xylostella* L. (Lepidoptera: Yponomeutidae)	Specialist
	Diaeretiella rapae McIntosh (Hymenoptera: Braconidae)	Generalist[a]	*Brevicoryne brassicae* L. (Hemiptera: Aphididae)	Specialist
	Hyposoter ebeninus Gravenhorst (Hymenoptera: Ichneumonidae)	Unknown[b]	*Pieris rapae* L. (Lepidoptera: Pieridae)	Specialist
Predator	*Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae)	Generalist	*Brevicoryne brassicae* L. *Myzus persicae* Sulzer (Hemiptera: Aphididae)	Specialist Generalist
	Episyrphus balteatus de Geer (Diptera: Syrphidae)	Generalist	*Brevicoryne brassicae* L. *Myzus persicae* Sulzer (Hemiptera: Aphididae)	Specialist Generalist

[a] *Although D. rapae parasitizes many aphid species (Pike et al. 1999), it is considered a specialist of aphids feeding on brassicaceous plants (Blande et al. 2004) and it is the main parasitoid of B. brassicae (Bukovinszky et al. 2008).* [b] *There is not much known about the number of host species of H. ebeninus. In the scientific literature P. rapae and P. brassicae are reported as host species (see e.g. Harvey et al. 2010).*

1.3.3 Selection of traits and environments

The multitrophic effects of plant resistance traits were studied under natural conditions in the field, as well as under controlled greenhouse and laboratory conditions. Due to legislative constraints on field testing of transgenic plants, transformed *A. thaliana* lines could only be studied in the laboratory. In the field, the population dynamics of the insects on the different white cabbage cultivars were determined by recording the abundance of each of the species on a weekly basis. The attraction of the natural enemies towards the white cabbage cultivars was determined by quantifying the parasitisation of herbivores and predator oviposition on the plants. In the greenhouse and the laboratory, the performance of the selected insect species on the white cabbage cultivars and/or on the wild-type and transformed *A. thaliana* lines

was determined. This was done by measuring their survival (or the percentage of successful parasitism in the case of parasitoids), development time and adult weight. For aphids, also the number of offspring was measured, in order to calculate the estimated intrinsic rate of population increase (r_m). The behaviour of the insect species was determined by recording their attraction towards a plant in two-choice bioassays.

1.4 Thesis outline

In the introductory **Chapter 2** the value of transgenic plants as components of Integrated Pest Management is discussed. The remainder of the thesis is divided into two main parts. The results from the fundamental studies on the multitrophic effects of plant resistance traits are discussed in Part I. In Part II, the step from basic ecology towards application in assessing the ecological effects of transgenic crops is made.

Part I: Multitrophic effects of plant resistance

Chapters 3-5 focus on the effects of intraspecific variation in resistance traits of *B. oleracea* on herbivores and their natural enemies. Four white cabbage cultivars that differ in direct and indirect resistance traits were used to study their effects on several herbivorous and carnivorous insect species under both greenhouse and field conditions.

In **Chapter 3,** the prey-mediated effects of GLS on aphid predators were studied under greenhouse conditions. Two predator species (*E. balteatus* and *C. carnea*) were fed either a GLS-sequestering specialist aphid (*B. brassicae*), or a non-sequestering generalist aphid (*M. persicae*) that excretes GLS in the honeydew, reared on four different white cabbage cultivars. Chemical analyses of host-plant phloem as well as the aphids feeding on this phloem were performed to correlate predator performance with plant and aphid chemistry.

Chapter 4 describes the relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance in the field. During two field seasons, the population dynamics of several herbivorous (*P. xylostella* and *B. brassicae*) and carnivorous (*E. balteatus*, *C. carnea*, *D. rapae* and *D. semiclausum*) insect species were recorded on four white cabbage cultivars throughout the season. To assess the relative importance of bottom-up and

top-down forces, chemical and morphological traits of the cultivars were quantified (bottom-up) and parasitisation of herbivores and predator oviposition on plants inoculated with a controlled number of herbivores was assessed (top-down).

Chapter 5 discusses whether the outcome of belowground–aboveground interactions can be affected by plant genotype under greenhouse conditions. Two white cabbage cultivars were selected, and the soil was inoculated with either nematodes or microorganisms and a sterilised soil acted as the control. Aboveground, aphid (*B. brassicae*) population development and parasitoid (*D. rapae*) fitness parameters were quantified to test whether aboveground insects were affected by belowground soil treatments, and whether plant genotype had an effect.

Chapters 6-8 discuss the effects of *A. thaliana* ecotypes that differ in their direct and indirect resistance traits and genetically transformed *A. thaliana* lines with enhanced direct or indirect resistance on herbivorous and carnivorous insect species under laboratory conditions.

In **Chapter 6** and **Chapter 7**, three *A. thaliana* ecotypes that differ in GLS content and one transformed *A. thaliana* line with modified concentrations of GLS were used to test the effects of GLS on 1) the performance of a specialist phloem-feeding aphid (*B. brassicae*) and the performance and behaviour of one of its predators (*E. balteatus*) and parasitoids (*D. rapae*) (**Chapter 6**); and 2) the performance of a generalist (*Spodoptera exigua* Hübner) and specialist (*P. rapae*) leaf-chewing herbivore and an associated parasitoid (*H. ebeninus*) (**Chapter 7**).

In **Chapter 8** the effects of modified VOC emission on the performance and behaviour of *B. brassicae* and the behaviour of two of its natural enemies, *E. balteatus* and *D. rapae*, were studied. Three *A. thaliana* ecotypes were transformed to emit one novel volatile compound and increased amounts of two endogenous compounds.

Part II: From basic ecology to application in transgenic crops

In **Chapter 9**, the main aim of the ERGO-project is addressed. In this chapter, the fundamental ecological knowledge on the interactions between plants and

insects reported in the previous chapters is applied to develop guidelines for environmental risk assessments of transgenic crops. The chapter presents guidelines for the COGEM to assess applications for field trials or commercial introduction of new transgenic crops, as submitted by breeding companies. This chapter is a combined effort of the three PhD-projects involved in this ERGO-project.

Finally, **Chapter 10** provides a summarising discussion on the fundamental and applied research findings. Here, the focus will be on the intraspecific differences in effects of direct and indirect plant resistance traits on different herbivorous and carnivorous insects, and how in-depth knowledge of these multitrophic interactions is essential in studying the ecology of transgenic crops. Future perspectives on the use of transgenic crops in agriculture and the value of the information from this thesis for assessing the ecological effects of new transgenic crops will be discussed.

2

Transgenic plants as vital components of Integrated Pest Management

Martine Kos, Joop J.A. van Loon, Marcel Dicke and Louise E.M. Vet

Published in *Trends in Biotechnology* 27: 621-627 (2009)

Abstract

Although Integrated Pest Management (IPM) strategies have been developed worldwide, further improvement of IPM effectiveness is required. The use of transgenic technology to create insect-resistant plants can offer a solution to the limited availability of highly insect-resistant cultivars. Commercially available insect-resistant transgenic crops show clear benefits for agriculture and there are many exciting new developments such as transgenic plants that enhance biological control. Effective evaluation tools are needed to ascertain that transgenic plants do not result in undesired non-target effects. If these conditions are met, there will be ample opportunities for transgenic plants to become key components of environmentally benign and durable pest management systems. Here we discuss the potential and challenges for incorporating transgenic plants in IPM.

2.1 Pest control in a changing world

Agriculture is continuously being adapted to meet the changing needs of society. The ever growing human population, combined with increases in soil and labour productivity, has dramatically affected crop cultivation. Over the last centuries, field enlargement, specialisation, mechanisation, increased genetic uniformity and higher crop plant density have contributed to the intensification of agriculture (Chadwick and Marsh 1993; Pimentel 2002). During this process, pest control largely relied on chemical pesticides because of their initial effectiveness, ease of use, relatively low costs and versatility (Dent 1995). However, the increasing use of pesticides in intensified agriculture has resulted in a number of problems, such as a build-up of insect resistance, environmental pollution and secondary pest outbreaks (Chadwick and Marsh 1993; Kortenhoff 1993; Pimentel 2002). The demand for environmentally friendly pest control techniques has steadily increased, particularly after publication of the book *Silent Spring* in 1962, which highlighted the detrimental effects of pesticides on the environment (Carson 1962).

Environmentally sound crop protection requires continuous innovation. Exciting new developments in biotechnology, in particular in genetic engineering, offer appealing possibilities for such innovations. Here, we discuss how biotechnology can provide opportunities for environmentally benign pest management systems.

2.2 Integrated Pest Management

To keep pests under control in modern agricultural systems without relying solely on pesticides, an approach called Integrated Pest Management (IPM) has been developed (Box 2.1). Although effective IPM strategies have been successfully developed for many crops worldwide, improvement of IPM programs will promote wide-spread application. A major improvement will come from new cultivars with high levels of resistance to pests. However, classical breeding for increased host plant resistance is time-consuming and labour-intensive and the desired beneficial trait can be linked to or associated with undesirable traits (linkage drag) (Dent 1995; Smith 2005). Furthermore, insect resistance in some cases compromises the efficiency of carnivorous arthropods, which are an important component of biological control (Hare 1992; Dicke 1999b). Classical breeding has rarely selected for traits that are beneficial to carnivores and instead might have led to a selection against these traits (Poppy and Sutherland 2004). In this opinion article, we propose the use

of biotechnology to develop insect-resistant transgenic plants as a valuable contribution to IPM.

2.3 Transgenic plants for enhanced direct resistance to pests

In genetic engineering approaches, plant traits can be modified by inserting DNA from a different species (Nap et al. 2003). The use of transgenic crops in agriculture has increased since they were first commercialised in the mid-1990s. In 2008, the global acreage of transgenic crops amounted to 125 million hectares, or 8% of the world's total agricultural land, representing a 74-fold increase since 1996. Insect-resistant crops comprised 15% of global transgenic crops in 2008 (James 2008).

All commercially available insect-resistant transgenic plants express genes coding for *Bacillus thuringiensis* (*Bt*) toxins that negatively influence the survival and development of a target herbivore (Aronson and Shai 2001; Chen et al. 2008). Genes coding for lectins, protease inhibitors, α-amylases or for other insecticidal products have also been successfully engineered into plants, resulting in negative effects on the survival and development of pest insects (Sarmah et al. 2004; Bi et al. 2006; Malone et al. 2008; Sadeghi et al. 2008). However, the only commercially available transgenic crops expressing genes other than *Bt* genes are cotton plants in China that express a serine protease inhibitor in combination with *Bt* (Malone et al. 2008). RNA interference (RNAi)-based control of insect pests uses transgenic plants that express double-stranded RNA (dsRNA), which reduces mRNA levels of a selected gene in the target herbivore upon feeding and consequently interferes with its development and survival (Baum et al. 2007; Price and Gatehouse 2008). RNAi-mediated knockdown of gene function has been reported in different insect orders, such as Lepidoptera, Hemiptera, Coleoptera, Diptera and Hymenoptera (Price and Gatehouse 2008), and can therefore be used for the control of various insect pests. For example, transgenic corn engineered to express dsRNA based on the sequence of a gene encoding an essential protein in the western corn rootworm (*Diabrotica virgifera virgifera*) resulted in a significant reduction in root damage by this coleopteran herbivore (Baum et al. 2007). Under the condition that the dsRNA introduced is very specific to certain insects to limit negative effects on non-target organisms, this is a very promising approach for transgenic pest control. However, the approach has only recently been developed and will need to be further optimised before it

Box 2.1 Integrated Pest Management

Integrated Pest Management (IPM) is defined as 'a durable, environmentally and economically justifiable system, in which damage caused by pests, diseases and weeds is prevented through the use of natural factors which limit the population growth of these organisms, if needed supplemented with appropriate control measures' (van Lenteren 1993). According to this definition, IPM is not just the combination of chemical control with another approach, but is based on the philosophy that natural pest control methods should be included before synthetic pesticides are used (van Lenteren 2008). The cornerstones of IPM are biological control, host plant resistance and cultural control. In biological control, a pest population is suppressed by enhancing the abundance or activity of indigenous natural enemies or by single or multiple introduction of natural enemies to the crop to obtain control of the pest (van Lenteren 2008). In host plant resistance, breeders use the ability of a plant to reduce its utilization as a host plant by a pest organism to select for crop cultivars with the highest resistance to pests and diseases. In cultural control, the environment is modified to make it less favourable for pest invasion, for example by crop rotation and tillage practices (Dent 1995). When a combination of biological control, host plant resistance and cultural control is insufficient, IPM can include the rational use of chemical pesticides (Dent 1995; Koul et al. 2004; Romeis et al. 2008a). Effective IPM strategies have been successfully developed for many crops world-wide and have resulted in reduced pesticide use, higher crop yields and economic value, as well as reduced economic risks for farm management owing to lower variation in the severity of pest problems (Dent 1995; Koul et al. 2004).

can be used on a wide scale (Price and Gatehouse 2008).

2.4 Transgenic plants that promote natural enemies vital for biological control

Herbivore damage induces the emission of plant volatile organic compounds (VOCs) that attract natural enemies of the damaging herbivore, a phenomenon also referred to as indirect resistance (Dicke and van Loon 2000; van Loon et al. 2000; van Poecke and Dicke 2004). In a new development for the application of genetic engineering, crop protection is enhanced by improving the effectiveness of biological control agents through modification of VOC

emission. Recently, increased VOC emission by the plant was genetically engineered as a novel trait. Laboratory and field studies demonstrated enhanced attraction of natural enemies of herbivores to these transformed plants, and even the repellence of herbivores (Aharoni et al. 2003; Kappers et al. 2005; Beale et al. 2006; Bouwmeester 2006; Schnee et al. 2006; Degenhardt et al. 2009) (Box 2.2).

Although transgenic crops exhibiting such indirect resistance are not yet commercially available, these plants are expected to become available to agriculture in the near future for the control of pest populations (Degenhardt et al. 2003; Poppy and Sutherland 2004; Kappers et al. 2005; Turlings and Ton 2006). There are still gaps in our knowledge regarding the exact mechanisms of indirect resistance of plants (Degenhardt et al. 2003), but we are convinced that genetically engineered crops with enhanced VOC emission to stimulate the recruitment of biological control agents will make a substantial contribution to environmentally sound pest control.

2.5 Incorporating transgenic plants in IPM

Transgenic crops that are toxic to herbivores and/or enhance the activity of carnivores will be important in feeding the growing world population. To date, transgenic crops have been used in conventional agriculture, in which pests are mainly controlled by pesticides, but their use in IPM has been limited. We believe that this is primarily the result of the gap that exists between biotechnology, in which a single solution to pest problems (reductionist approach) is the focus, and IPM, in which an optimal combination of different pest control techniques (holistic approach) is the aim. Insect-resistant transgenic crops have been advertised as being the sole solution to pest problems, whereas IPM practitioners have found that a single solution is rarely sufficient and, moreover, is not durable. Indeed, current transgenic crops based on *Bt,* can solve lepidopteran or coleopteran pest problems, but secondary pests such as aphids can arise when competition from the target pest is relieved (Cloutier et al. 2008).

Although few crop protectionists view transgenic plants as a (potential) component of IPM, we believe that current and future transgenic plants have the greatest potential when grown under IPM regimes. It has already been demonstrated that currently available insect-resistant transgenic plants are compatible with other IPM approaches, such as biological, chemical and cultural control (Smith 2005; Romeis et al. 2008b).

Box 2.2 Genetic engineering of VOC emission: several case studies

Several studies have shown that increasing the constitutive emission of plant VOCs through genetic engineering can attract natural enemies to these transgenic plants, and even repel herbivores. In one study, metabolic engineering of terpenoids, previously shown to be important compounds in attracting carnivorous enemies of pest herbivores, was enhanced in *Arabidopsis thaliana* plants. The transgenic plants constitutively emitted two new terpenoids, the sequiterpene (3S)-(*E*)-nerolidol and the homoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene [(*E*)-DMNT], not emitted before. As a result predatory mites were attracted to these plants, whereas they were not attracted to wild-type plants (Kappers et al. 2005). In another study, *A. thaliana* was genetically engineered to produce several sesquiterpenes such as (*E*)-β-farnesene and (*E*)-α-bergamotene. After previous oviposition experience in their host caterpillars in the presence of the sesquiterpene-emitting transgenic plants, parasitoid wasps of the species *Cotesia marginiventris* were significantly attracted to the transgenic plants in an olfactometer test (Schnee et al. 2006). In a third case, *A. thaliana* was engineered to emit the sesquiterpene (*E*)-β-farnesene, and parasitoid wasps of the species *Diaeretiella rapae* showed a significant increase in time spent on transgenic compared to wild-type plants. Furthermore, the generalist aphid *Myzus persicae* was repelled by the transgenic plants (Beale et al. 2006). Also in other studies, elevated VOC emissions resulted in herbivore repellence, as shown for *M. persicae* offered transgenic *A. thaliana* emitting the terpenes linalool and nerolidol (Aharoni et al. 2003). In the first reported field experiment using a transgenic crop with increased VOC emissions, maize plants were transformed to emit the naturally active substance (*E*)-β-caryophyllene from their roots. This compound attracts nematodes that attack and kill larvae of the western corn rootworm (*Diabrotica virgifera virgifera*). American maize lines, in contrast to European maize lines, do not emit (*E*)-β-caryophyllene. A non-emitting maize line was transformed with an (*E*)-β-caryophyllene synthase gene from oregano under the control of a constitutive promoter, resulting in constitutive emission of this sesquiterpene. In rootworm-infested field plots in which nematodes were released, the (*E*)-β-caryophyllene-emitting plants suffered significantly less root damage and had 60% fewer adult beetles emerging than untransformed, non-emitting lines (Degenhardt et al. 2009).

Below, the potential and challenges for integration of transgenic plants in IPM are discussed.

2.5.1 Potential for incorporating transgenic plants into IPM

Transgenic insect resistance has several advantages over insect resistance achieved through classical breeding. New varieties can be produced considerably faster by genetic engineering than by conventional breeding. Furthermore, the level of resistance in transgenic plants is potentially higher than resistance levels naturally occurring within the gene pool of a plant species because genes from different sources can be introduced and linkage drag is minimized (Smith 2005; Kennedy 2008).

The benefits of growing *Bt* crops such as cotton, corn, potato and rice have been well described. Pests, mainly Lepidoptera such as the European corn borer (*Ostrinia nubilalis*) and the cotton bollworm (*Helicoverpa armigera*), have been successfully controlled in *Bt* crops, resulting in increases in crop yield and economic value, while at the same time allowing drastically reduced use of pesticides aimed at target pests in these crops (Shelton et al. 2002; Smith 2005; Fitt 2008). In addition, natural enemies of pests have profited from the decrease in the application of broad-spectrum pesticides, thus increasing their contribution to natural pest control in these crops (Sisterson et al. 2007; Romeis et al. 2008b). Moreover, the prolonged development time for a herbivore after ingesting insecticidal toxins with sub-lethal effects can increase its exposure to carnivores according to the so-called slow-growth, high-mortality hypothesis (Williams 1999; Cornelissen and Stiling 2006). This might further enhance the effectiveness of pest control through the action of carnivores. Because *Bt*-toxins are contained within the plant, exposure of non-target organisms that do not feed on the plant itself is significantly reduced compared to exposure resulting from pesticide spraying (Jouanin et al. 1998). Moreover, pests that feed on plant parts typically not reached by pesticide sprays, such as stem borers that feed within the plant stem, are exposed to the transgenic *Bt*-toxins (Jouanin et al. 1998; Thies and Devare 2007; Romeis et al. 2008a). Furthermore, because toxins are expressed in the plant tissue, insects are exposed for the entire infestation period, including the most vulnerable stages in their development (Jouanin et al. 1998; Thies and Devare 2007; Romeis et al. 2008a). It should also be noted that specific *Bt*-toxins are only toxic to a limited range of species within one or two insect orders, and transgenic resistance of *Bt*-plants is therefore much more specific for the

target insect than many pesticides (de Maagd et al. 1999).

Biological control of pests might be facilitated to an even greater extent by altering VOC emission as discussed above. Through selective changes in VOC composition and emission rate, plants can more strongly attract carnivorous natural enemies of herbivores, whereas other plant resistance functions are not compromised (Degenhardt et al. 2003). Because pest resistance to a biological control agent is less likely to evolve (Bale et al. 2008), the risk of the pest becoming insensitive to transgenic resistance based on enhanced carnivore attraction is very small. Furthermore, attraction of several carnivorous species, including generalists that feed on a range of prey species, is likely to prevent secondary pest outbreaks.

VOCs are not only used by carnivores to locate their prey, but can also repel herbivores, presumably by indicating the presence of potential competitors and natural enemies (Dicke and van Poecke 2002; Sanchez-Hernandez et al. 2006). Several studies reported these repellent effects of VOCs on feeding and oviposition behaviour of different herbivore species, including the tobacco hornworm *Manduca sexta*, the leaf beetle *Cerotoma ruficornis* and the diamondback moth *Plutella xylostella* (Heil 2004; Sanchez-Hernandez et al. 2006; Yang 2008). If VOC emission is engineered in such a way that the transgenic plant not only attracts natural enemies of pest herbivores, but also repels certain herbivores, this would further increase the benefits of such transgenic plants.

2.5.2 Challenges of growing transgenic crops within IPM

If transgenic plants are to become components of IPM, several challenges need to be dealt with. One of the major threats to the effectiveness of genetic engineering is the development of insensitivity to the toxin by the pest population arising from the intense selection pressure the transgenic crops imposes (Bravo and Soberon 2008; Ferré et al. 2008). Resistance to *Bt*-sprays has already been reported (Ferré et al. 2008) and artificial selection for insensitivity in the laboratory during 6-8 generations has resulted in 80-100-fold lower sensitivity to *Bt*-toxins, suggesting that substantial reduction in levels of plant resistance in *Bt* crops might occur (Tabashnik et al. 2008). Therefore, it is important to implement resistance management strategies when cultivating transgenic crops. For example, by offering a refuge from exposure to the toxin to allow the survival of susceptible insects within the population, development of resistance could be prevented or slowed down

(Bravo and Soberon 2008; Ferré et al. 2008). Other approaches are the temporal rotation of cultivars that express different insecticidal proteins and the stacking of multiple genes (Bravo and Soberon 2008; Ferré et al. 2008; Tabashnik et al. 2008).

Another risk associated with growing transgenic plants is the outbreak of secondary pests caused by elimination of the major pest combined with a decrease in the use of broad-spectrum pesticides (Romeis et al. 2008a). For example, aphids and thrips are not targeted by any of the available *Bt*-toxins and could increase in abundance in *Bt* crops. Furthermore, insect-resistant transgenic crops can only be used as a preventive measure. If an unanticipated pest not targeted by the insecticidal protein arrives during the growing season, the crop could become highly vulnerable to this newcomer species in the absence of other control measures (Kennedy 2008).

However, an indirect effect of the use of transgenic crops, the reduced application of broad spectrum pesticides, will lead to increased abundance of natural enemies (Sisterson et al. 2007; Romeis et al. 2008b) and might reduce the number or severity of secondary pest outbreaks and decrease the risk of insensitivity development because such enemies will also attack the target herbivores. Furthermore, active release of biological control agents during any secondary pest outbreak or when insensitivity has developed could reduce the probability of pest outbreaks. Therefore, although these risks are often regarded as disadvantages of transgenic crops, these risks disappear when transgenic crops are incorporated in an IPM context.

When biological control of the secondary or unanticipated pests is not sufficiently fast or efficient, it might be necessary to apply specific pesticides to prevent crop loss. Although IPM aims at preventing this situation, the rational use of selective pesticides is a legitimate strategy within IPM (Dent 1995; Koul et al. 2004).

Transgenic plants with altered VOC emissions pose a different set of challenges because VOCs play roles in different multitrophic interactions (Dicke and Vet 1999; Dicke and van Loon 2000; Dicke et al. 2003). VOCs not only repel herbivores as outlined above, but might also attract certain herbivores by indicating the presence of a suitable host plant. For example, studies have shown that plants damaged by herbivory are more attractive to certain herbivores, such as the tobacco flea beetle (*Epitrix hirtipennis*), the fall armyworm (*Spodoptera frugiperda*) and curculionid beetles, than undamaged plants (Heil 2004; Carroll et al. 2006; Halitschke et al. 2008). Because

attraction of additional herbivore species to transgenic plants with altered VOC emissions is undesirable, detailed information about the effects of VOCs on potential pest herbivores should be obtained before this approach is implemented in practice.

Selection of a herbivore-inducible promoter to ensure emission of increased amounts of VOCs only after herbivore attack, is an important prerequisite that prevents carnivores from being attracted to the plant in the absence of their prey. Furthermore, because natural enemies are capable of learning, they might learn that the constitutive signal does not indicate the presence of their prey and would consequently ignore the signal (Papaj et al. 1994; Degenhardt et al. 2003; Poppy and Sutherland 2004). This would decrease the pest-control efficiency of such transgenic plants.

2.6 Effects of transgenic crops on non-target organisms

The effects of transgenic plants on non-target organisms are an important aspect when considering the use of transgenic crops within IPM. Non-target organisms, such as carnivores or pollinators, can be exposed to the transgenic product in several ways (Fig. 2.1). Only plants that do not negatively influence important non-target organisms, or at least show a lower impact than currently observed for other control measures, hold promise within an IPM framework. Therefore, it is particularly important that potential non-target effects of transgenic crops are carefully assessed.

However, there is a lack of clear guidelines for the testing of non-target effects of transgenic plants, even though European Union legislation requires testing of these effects (Box 2.3). The few available studies focused on improving the assessment of non-target effects proposed a stepwise (tiered) approach for the testing of non-target effects of transgenic plants expressing insecticidal proteins (Dutton et al. 2003; Poppy and Sutherland 2004; Andow and Zwahlen 2006; Birch et al. 2007; Charleston and Dicke 2008) (Fig. 2.2). Although methods for testing non-target effects are well established for laboratory studies (the 1st tier in Fig. 2.2) (Dutton et al. 2003; Charleston and Dicke 2008), detailed methods for (semi-)field experiments to validate laboratory results are lacking, despite their crucial role in establishing whether results observed in the laboratory or greenhouse are indeed transferable to the agro-ecosystem.

This indicates that there is an urgent need for universally applicable methods for non-target testing of different insect-resistant transgenic plants.

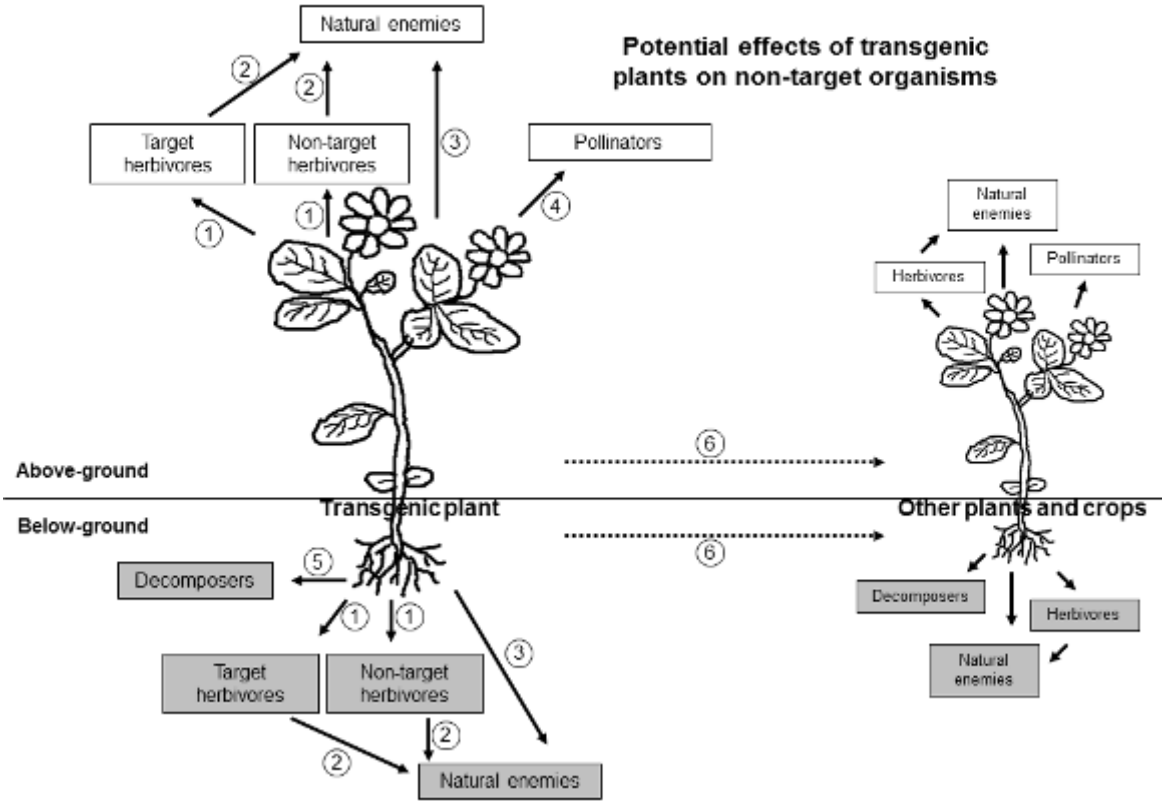


Fig. 2.1 *Potential effects of transgenic plants on non-target organisms (partly based on information from Groot and Dicke 2002; Scholte and Dicke 2005; Thies and Devare 2007; Charleston and Dicke 2008). The transgenic plant shown here represents plants either expressing insecticidal proteins or with altered VOC emissions. The different possible effects of transgenic plants on target and non-target organisms are shown. (1) Direct effects on target and non-target herbivores arise (a) through plant feeding, (b) in response to VOCs, which can act as oviposition and feeding stimulants or as repellents, (c) by contact with soil exposed to transgenic products through plant decomposition or root exudation. (2) Host- or prey-mediated effects on natural enemies are indirect effects that arise through feeding on host or prey items that themselves fed on the transgenic plant. (3) Direct effects on natural enemies of herbivores arise (a) from feeding on plant material, such as nectar and pollen, (b) in response to VOCs, (c) by contact with soil exposed to the transgenic product. (4) Direct effects on pollinators arise from feeding on nectar and pollen or in responding to VOCs. (5) Direct effects on decomposers arise from feeding on plant material or coming into contact with soil exposed to the transgenic product. (6) Effects on other plants and crops arise, for example, by transfer of pollen or by contact with soil exposed to the transgenic product. Exposure to VOCs from neighbouring plants can prime other plants for increased resistance to herbivores (Baldwin et al. 2006; Turlings and Ton 2006), which in turn could influence organisms associated with the plant or crop.*

Box 2.3 Guidelines for non-target testing of transgenic plants

European Union (EU) regulatory bodies require detailed environmental risk-assessments before they permit the release of a transgenic crop into the environment. Transgenic plants are considered to be new entities in the EU, so risk-assessments for transgenic crops differ from standard risk assessments for conventional plant protectants and require evaluation of potential adverse effects of deliberate release of transgenic crops on the environment (European Community 2001). However, the EU does not provide guidelines with regard to how such ecological risk assessments should be carried out. No instructions are provided on what information should be presented, what experiments should be carried out and what non-target organisms should be tested. Furthermore, the appropriate baseline against which the ecological significance of any non-target effects should be assessed is not expressed explicitly.

Despite this lack of clear guidelines, the effects of *Bt*-plants on non-target arthropods have been extensively tested (see *e.g.* reviews by Groot and Dicke 2002; Clark et al. 2005; Romeis et al. 2006; Thies and Devare 2007). However, analysis of these non-target studies revealed important gaps in our knowledge because variable and conflicting results are presented for non-target tests, even for the same non-target species, most likely resulting from the use of different techniques or the examination of different parameters (Bruinsma et al. 2003; Charleston and Dicke 2008). Furthermore, an integrated approach combining above- and belowground systems does not yet exist, but both scenarios should be included in the risk-assessment because plants are important links between organisms that live aboveground and belowground (van der Putten et al. 2001; Bruinsma et al. 2003; Bezemer and van Dam 2005). In addition, most non-target studies have focused on effects on the fitness of the non-target organisms, but effects on their behaviour should also be considered (Poppy and Sutherland 2004), because these might, for instance, limit any negative effects of transgenic plants on the non-target organism under field conditions. Ignoring behavioural effects of non-target organisms might thus lead to a distorted view of the potential environmental effects of transgenic plants.

To prevent incorrect assessment of non-target effects, detailed tools for the evaluation of potential effects aboveground and belowground will need to be developed soon, not only for plants expressing insecticidal proteins, but also for plants with enhanced VOC emissions.

2.7 Future perspectives

As discussed above, transgenic plants have the potential to become an integral component of IPM. There are excellent opportunities to avoid the potential ecological risks related to transgenic crops, such as secondary pest outbreaks and pest resistance, by incorporating insect-resistant transgenic plants in IPM systems.

Ongoing developments in biotechnology should provide exciting new possibilities for the control of pest populations in an environmentally friendly manner. It is anticipated that future generations of transgenic plants will be able to enhance biological control through modification of herbivore-induced VOC blends that in turn will attract natural enemies. Other promising developments include insect-resistant transgenic crops that express genes coding for insecticidal proteins other than *Bt* or that rely on RNAi-based pest control (Malone et al. 2008; Price and Gatehouse 2008).

Stacking of multiple genes can also provide increased protection against multiple harmful organisms, such as herbivores, diseases and weeds, and has the added advantage of reducing the risk of the emergence of herbivore resistance (Ferré et al. 2008; Tabashnik et al. 2008). The acreage of these so-called pyramided transgenic crops is already increasing (James 2008). In the future, stacking genes that confer direct toxicity against herbivores and indirect attraction of carnivores might further enhance the efficiency of pest control and further reduce the risk of pest resistance.

To successfully incorporate biotechnology in IPM, the reductionist and holistic approaches that these fields represent need to be integrated. To achieve this, a new generation of scientists should be trained in both areas. Educational and research programmes are necessary to inform this new generation about the possibilities in both approaches to crop protection. We anticipate that bridging the gap between the two worlds will result in the view that transgenic crops are vital components of IPM, thus opening new perspectives for environmentally friendly pest control.

2.8 Acknowledgements

This work was supported by a grant from the Netherlands Organisation for Scientific Research (NWO) under the ERGO programme (NWO number 838.06.010).

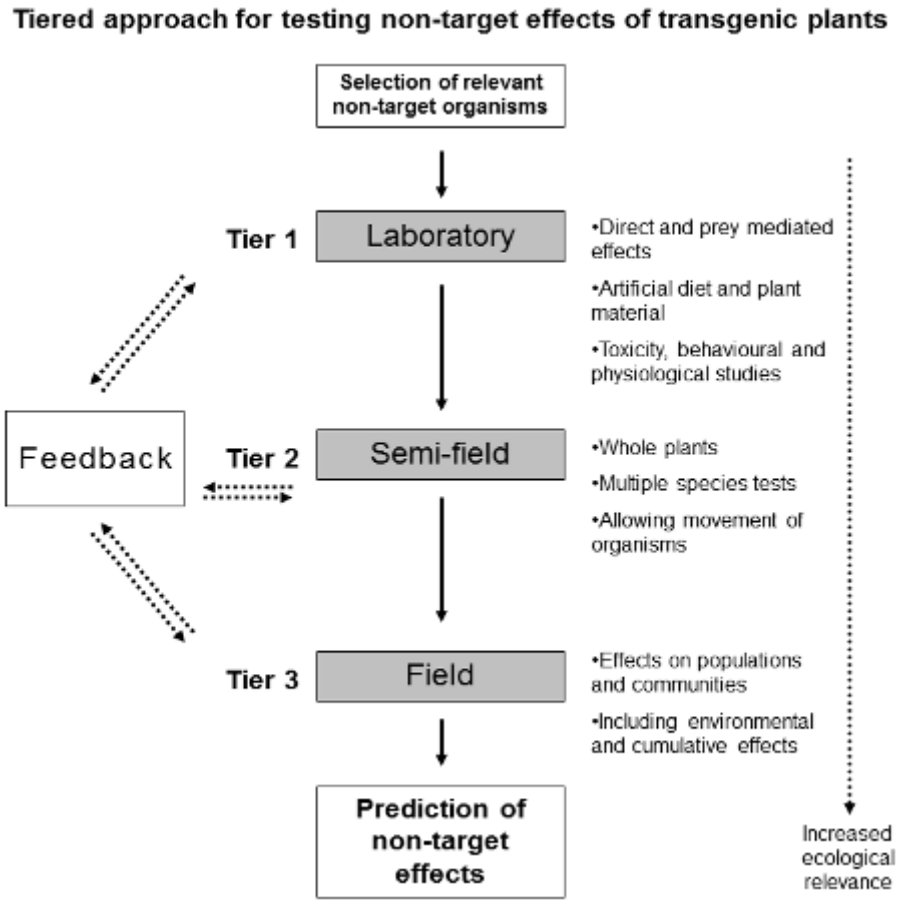


Fig. 2.2 *Stepwise (tiered) approach for testing the effects of transgenic plants on non-target organisms (summarised from Dutton et al. 2003; Poppy and Sutherland 2004; Andow and Zwahlen 2006; Birch et al. 2007; Charleston and Dicke 2008). The relevant non-target species have to be selected, after which the assessment starts in the laboratory and increases in complexity and realism towards semi-field and field set-ups, with knowledge gained in previous steps used in subsequent steps. However, the tiers should not be just considered as sequential steps in a linear approach, because feedback between the tiers is necessary during the assessment. If necessary, results from one level should be re-examined at another level to fill existing and emerging knowledge gaps. Only when the effects of transgenic plants on non-target organisms have been tested at all levels can a reliable prediction of the non-target effects of the transgenic crop be made.*

3

Prey-mediated effects of glucosinolates on aphid predators

Martine Kos, Patrick Kabouw, Rozemarijn Noordam, Koen Hendriks, Louise E.M. Vet, Joop J.A. van Loon and Marcel Dicke

Published in *Ecological Entomology* 36: 377-388 (2011)

Abstract

Plant resistance against herbivores can act directly (*e.g.* by producing toxins) and indirectly (*e.g.* by attracting natural enemies of herbivores). If plant secondary metabolites that cause direct resistance against herbivores, such as glucosinolates (GLS), negatively influence natural enemies, this may result in a conflict between direct and indirect plant resistance.

Our objectives were 1) to test herbivore-mediated effects of GLS on the performance of two generalist predators, the marmalade hoverfly (*Episyrphus balteatus*) and the common green lacewing (*Chrysoperla carnea*) and 2) to test whether intraspecific plant variation affects predator performance.

Predators were fed either *Brevicoryne brassicae*, a GLS-sequestering specialist aphid that contains aphid-specific myrosinases, or *Myzus persicae*, a non-sequestering generalist aphid that excretes GLS in the honeydew, reared on four different white cabbage cultivars. Predator performance and GLS concentrations and profiles in *B. brassicae* and host-plant phloem were measured, a novel approach as previous studies often measured GLS concentrations only in total leaf material.

Interestingly, the specialist aphid *B. brassicae* selectively sequestered GLS from its host plant. The performance of predators fed this aphid species was lower than when fed *M. persicae*. When fed *B. brassicae* reared on different cultivars, differences in predator performance matched differences in GLS profiles among the aphids.

We show that not only the prey species, but also the plant cultivar can have an effect on the performance of predators. Our results suggest that in the tritrophic system tested, there might be a conflict between direct and indirect plant resistance.

3.1 Introduction

Plants have two different resistance mechanisms against herbivorous insects, namely direct and indirect resistance. Direct resistance affects herbivores through physical (*e.g.* thorns) or chemical (*e.g.* toxins or digestibility reducers) plant traits. Indirect resistance influences the effectiveness of natural enemies of herbivores through *e.g.* the emission of volatile herbivore-induced secondary plant metabolites that attract the natural enemies (Karban and Baldwin 1997; Dicke and Baldwin 2010). Secondary plant metabolites that mediate direct resistance, however, may affect not only herbivores. They can also negatively influence natural enemies of herbivores, either directly through feeding on the herbivore that contains the secondary metabolites, or indirectly through reduced host or prey quality (Francis et al. 2001a; Harvey 2005; Ode 2006). This may result in a conflict between direct and indirect plant resistance (Sznajder and Harvey 2003; Gols and Harvey 2009). With some exceptions (see Sznajder and Harvey 2003; Gols and Harvey 2009 and references therein), direct and indirect resistance strategies are mostly studied independently, disregarding the potential evolutionary conflict between them.

To study effects of secondary plant metabolites on natural enemies of herbivores, we focus on glucosinolates (GLS). GLS are a group of secondary metabolites that are characteristic for the plant family Brassicaceae and that show considerable variation in concentrations between and within species of this family (Gols et al. 2008b; van Dam et al. 2009). When herbivores damage plant tissues, GLS become exposed to myrosinases, enzymes that are spatially separated from GLS in intact plant tissues. Myrosinases degrade GLS to compounds such as (iso)thiocyanates and nitriles, depending among others on the side chain of the GLS. These hydrolysis products are toxic to a wide variety of insects (Bones and Rossiter 2006; Halkier and Gershenzon 2006). GLS and their breakdown products have negative effects on generalist insect herbivores *e.g.* by deterring feeding or decreasing survival (Halkier and Gershenzon 2006; Hopkins et al. 2009). Specialist herbivores of brassicaceous plants, in contrast, have evolved special adaptations to detoxify GLS (Ratzka et al. 2002; Wittstock et al. 2004), and use these compounds as feeding or oviposition stimulants (van Loon et al. 1992; Miles et al. 2005). GLS also affect organisms on higher trophic levels. Several specialist natural enemies of herbivores are attracted by volatile breakdown products of GLS (Bradburne and Mithen 2000; Blande et al. 2007; Mumm et al. 2008). GLS from the host plant may, however, also negatively affect natural enemies that

feed on GLS-containing herbivores (Francis et al. 2001b; Vanhaelen et al. 2002; Sznajder and Harvey 2003). Some specialist herbivores sequester GLS from the host plant and use these as a defence against their natural enemies (Müller et al. 2001; Bridges et al. 2002; Müller and Brakefield 2003). Sequestration of GLS by the herbivore *Brevicoryne brassicae* L. (Hemiptera: Aphididae), for example, has been shown to decrease the performance of generalist predators such as ladybirds (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008).

The objectives of this study were 1) to test herbivore-mediated effects of GLS on the performance of two generalist predators, the marmalade hoverfly (*Episyrphus balteatus* de Geer; Diptera: Syrphidae) and the common green lacewing (*Chrysoperla carnea* Stephens; Neuroptera: Chrysopidae) and 2) to test whether intraspecific plant variation affects predator performance. The predators were fed either *B. brassicae* or *Myzus persicae* Sulzer (Hemiptera: Aphididae) reared on four white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba*) cultivars that have previously been shown to differ in their GLS profiles and resistance to herbivores (Broekgaarden et al. 2008; Poelman et al. 2008b; Kabouw et al. 2010a). *Brevicoryne brassicae* and *M. persicae* differ in the concentration of GLS, as well as in the presence of toxic hydrolysis products of these GLS. *Brevicoryne brassicae* sequesters GLS from the phloem of its host plant and contains aphid-produced myrosinases (Jones et al. 2001; Francis et al. 2002). Upon tissue damage by carnivores, the sequestered GLS in *B. brassicae* come into contact with aphid myrosinases, causing the formation of toxic breakdown products (Bridges et al. 2002; Kazana et al. 2007). *Myzus persicae* does not sequester GLS, but excretes them in the honeydew, and does not contain myrosinases that could break down the GLS that are present in the gut into toxic breakdown products (Francis et al. 2001b). Aphid predators will therefore encounter high concentrations of toxic GLS breakdown products when feeding on *B. brassicae*, but not when feeding on *M. persicae*. We hypothesized that, based on the difference in concentrations of GLS and their breakdown products, the performance of *C. carnea* and *E. balteatus* will be lower when fed *B. brassicae* than when fed *M. persicae*. Furthermore, we expect that variation in GLS composition among the host-plant cultivars would affect the GLS composition of the aphids feeding on these cultivars, as well as the possible formation of breakdown products (which depends on the side chain identity, see above), and thereby the performance of the natural enemies feeding on these aphids.

Effects of sequestration of GLS by *B. brassicae* on aphid predators (mainly lady bird beetles and hoverflies) have been tested before (see *e.g.* Francis et al. 2001b; Kazana et al. 2007; Pratt 2008). Previous studies often (1) only reported the GLS concentrations in the host plant, and not in the prey insect itself, or (2) linked GLS concentrations in total leaf material to GLS concentrations in the aphid, whereas aphids do not chew leaf tissue, but feed on phloem sap exclusively. Our study presents novel data because we have analysed GLS concentrations as well as profiles in the phloem sap and the aphids feeding on this phloem sap. This resulted in a detailed comparative analysis of the effects of GLS sequestration of a specialist aphid on two of its main predator species, of which one, the green lacewing, has not yet been the subject of testing effects of GLS on its performance. We discuss the effects of intraspecific plant variation in GLS composition on aphid-predator interactions in the context of a possible conflict between direct and indirect plant resistance.

3.2 Materials and methods

3.2.1 Plants and insects

We used four white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba*) cultivars: Christmas Drumhead and Badger Shipper (Centre for Genetic Resources, CGN, Wageningen, The Netherlands), representing open pollinated cultivars, and Lennox and Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands), representing more recently cultivated F1 hybrids. Plants were cultivated in a greenhouse compartment at 20 ± 2 °C, 60-70% relative humidity (RH) and a 16:8 h light:dark (L:D) photoregime. When the light dropped below 200 W m^{-2} during the photoregime, supplementary illumination was provided by sodium lamps (SON-T Philips, Eindhoven, The Netherlands). Seeds were germinated on peat soil (Lentse potgrond, no. 4, Lent, The Netherlands) and after 8 days, individual seedlings were transferred to the same peat soil in 1.45 L pots. All plants were watered daily and were fertilised by applying Kristalon Blauw (Hydro Agri, Rotterdam, The Netherlands) (N-P-K-MgO) 19-6-20-3 micro (2.5 mg L^{-1}) to the soil twice a week from the age of four weeks onwards. Six-week-old plants were used in the experiments.

Brevicoryne brassicae and *Myzus persicae* were reared on the four white cabbage cultivars and Brussels sprouts (*Brassica oleracea* L. convar. *gemmifera* cv. Cyrus) in greenhouse compartments at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime. Fresh plants were provided on a weekly basis

and plants were watered every other day. The *M. persicae* and *B. brassicae* cultures were originally collected from *B. oleracea* in the vicinity of Wageningen (The Netherlands) in 2004 and 2008 respectively.

Episyrphus balteatus pupae and *C. carnea* eggs were provided by Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands) and kept in a greenhouse compartment at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime. *Episyrphus balteatus* pupae were kept in gauze cages (67 x 50 x 67 cm) and adults emerging from the pupae were provided with water, sugar, and bee-collected pollen provided by Koppert Biological Systems. Females were allowed to lay eggs on Brussels sprouts plants infested with either *B. brassicae* or *M. persicae.* After egg hatch, neonate larvae of both species were used in the experiments. (Note: it has been recently discovered that *C. carnea* is actually a complex of many cryptic sibling species (Henry et al. 2002), and the specific *C. carnea* we used was identified by Koppert Biological Systems as the sibling species *C. affinis*).

3.2.2 Aphid performance

All experiments were performed in a greenhouse compartment at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime. Aphid performance on the four white cabbage cultivars was assessed, because it is known that the performance of a natural enemy is often positively correlated with the performance of its host or prey (Benrey et al. 1998; Sznajder and Harvey 2003). Data on performance of *B. brassicae* on the four white cabbage cultivars were derived from Broekgaarden et al. (2008). We assessed performance of *M. persicae* according to the protocol of Broekgaarden et al. (2008) to allow for direct comparisons of performance of both aphid species on the selected cultivars. Of each cultivar, 15 six-week old plants were infested with *M. persicae* by placing one neonate nymph on each of five older leaves. Plants were placed individually in gauze nets and distributed randomly over the greenhouse. Plants were watered every other day and fertilised weekly in the same way as described under *Plants and insects*. Nymphs were monitored daily to estimate their development time (number of days between birth and reproduction) and nymphal survival was scored on day 11, the day by which most of the individuals had reproduced. From day 11 onwards, the number of aphids on each plant was recorded twice a week until day 34 of the experiment. Aphid multiplication factor was calculated by setting the number of aphids at day 11 of the experiment to 1.

3.2.3 GLS analysis

In order to examine GLS concentrations in *B. brassicae* feeding on different cultivars, and to link this to GLS concentrations in the phloem of these cultivars, we infested 10 six-week old plants of each cultivar with hundreds of neonate nymphs by allowing adults to reproduce on the plant for 24 h, after which the adults were removed from the plants. When nymphs reached the third instar, half of the aphids on each plant were collected for GLS analysis, resulting in 10 replicates of several hundred nymphs per cultivar. At the same time, we collected phloem exudate from the third youngest fully expanded leaf of each of these 10 plants per cultivar. For phloem collection we followed the procedure of Bezemer et al. (2005) with minor adaptations: we used 2 ml 8mM EDTA solution, initially placed the petiole of the leaf for 5 min in the EDTA solution to remove any plant chemicals from the incision, and afterwards placed the petiole for 2 hours in a new vial with 2 ml EDTA solution. Our method inherently sampled a small amount of mesophyll fluids mixed with the phloem sap. Therefore, if we refer to phloem sap we mean phloem sap plus these potential contaminants from the mesophyll. Subsequently, the leaf was dried at 80 °C for 3 days and its dry weight was measured on a balance (Mettler-Toledo PM200, Tiel, The Netherlands). When the aphids reached adulthood, the remaining aphids were collected for GLS analysis, resulting in 10 replicates of several hundred adult aphids per cultivar. At the same time, additional phloem samples were taken from the fourth youngest fully expanded leaf of each of these 10 plants per cultivar. GLS concentrations were analysed separately for third instar nymphs and adult aphids to allow for investigation of differences in GLS sequestration between nymphs and adult aphids.

All samples were frozen at -20 °C immediately after collection. Aphid samples were freeze-dried, weighed and ground to a fine powder. Approximately 50 mg of the ground material of third instar nymphs and 100 mg of adult aphids was weighed into a micro-centrifuge tube. GLS were extracted and purified by using the methods of van Dam et al. (2004) and Kabouw et al. (2010a) and GLS content was assessed by high-performance liquid chromatography (HPLC). GLS detection was performed with a photodiode array detector with 229 nm as the integration wavelength. Different concentrations of sinigrin (Acros, Morris Plains, New Jersey, USA) were used as an external standard. The correction factors at 229 nm from Buchner (1987) and the European Community (1990) were used to calculate the concentrations of the GLS. We identified desulfoGLS peaks by comparison of

HPLC retention times and ultraviolet spectra with standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology, Jena, Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels, Belgium, code BCR-367 R).

To extract GLS from the phloem, we used a modified protocol. One ml from the collected 2 ml of EDTA solution was used for GLS extraction. The solution was boiled in a water bath at 70 °C, subjected to an ultrasonic bath for 15 min to inactivate myrosinase activity, and afterwards transferred directly onto a Sephadex column. To concentrate the samples, after elution the freeze-dried eluate was resuspended in 100 µl instead of 1000 µl water.

3.2.4 Predator performance

Performance of predators was tested in no-choice situations. We assessed the performance of both *E. balteatus* and *C. carnea* fed either *M. persicae* or *B. brassicae* reared on each of the four white cabbage cultivars, thus creating eight prey species x plant cultivar combinations. For each predator species 40 individual predator larvae were monitored for each of the eight combinations, set up in a randomised design. Neonate larvae were transferred individually to a Petri dish (ø 9 cm) with filter paper on the bottom by using a fine paintbrush. Larvae were fed *ad libitum* with aphids of mixed instars (representing the natural situation in which aphids occur in colonies of mixed ages) from the selected prey treatment that were provided together with a leaf fragment of the corresponding plant cultivar. Each day the prey items and leaf fragments were replaced. Survival rate, larva-to-adult development time, pupal fresh weight, sex, adult dry weight, head width, and wing length were measured. Adult dry weight was obtained by weighing adults that had been dried to constant weight at 80 ˚C for 3 days on a microbalance (Sartorius CP2P, Göttingen, Germany). Head width and wing length were measured using a stereo microscope (Olympus SZX12), attached to a digital camera (Euromex CMEX-1) and the program Image Focus (version 1.0).

3.2.5 Statistical analysis

Statistical analyses were performed using SPSS for Windows (15th edition, Chicago, Illinois, USA), unless indicated otherwise. Nymphal survival of *M. persicae* was analysed by logistic regression in Gen Stat (12th edition, VSN International, UK). Development time was log-transformed to obtain normality, and analysed by ANOVA and post-hoc LSD-test for cultivar comparisons.

Aphid multiplication factor was log-transformed and repeated measures ANOVA and post-hoc LSD-test for cultivar comparisons were used to assess the impact of different cultivars on the multiplication factor over time. Time was considered a within-subjects factor and cultivar a between-subjects factor. Differences in aphid multiplication factors over time between *B. brassicae* and *M. persicae* were analysed by repeated measures ANOVA.

Differences in indole, aliphatic and total GLS concentrations in phloem and aphids among the cultivars were analysed by Kruskal-Wallis H-tests, as assumptions on normality were violated. Mann-Whitney U tests with a Bonferroni correction for the number of comparisons (six) were used to compare the mean differences between the groups. Differences in indole, aliphatic and total GLS concentrations between both aphid developmental stages and between both phloem sampling dates were analysed by Mann-Whitney U tests. Correlations between indole, aliphatic and total GLS concentrations in the phloem and the concentrations of these compounds in the aphids feeding on those plants were tested with Spearman's correlation test.

To analyse GLS profiles of phloem and aphids, we used projection to latent structures-discriminant analysis (PLS-DA) and partial least squares projections to latent structures (PLS), in SIMCA-P (12^{th} edition, Umetrics, Umeå, Sweden) (Eriksson et al. 2006). PLS-DA is a multivariate discriminant analysis that we used to test if GLS profiles in the phloem of the different cultivars differed significantly and if GLS profiles in the aphids feeding on these cultivars also differed significantly. PLS is a multivariate method for regression analysis that we used to test the relationship between GLS profiles in phloem and GLS profiles in aphids feeding on those plants. To pre-process data, GLS concentrations were log-normalised, mean-centred and scaled to unit variance.

Survival and sex ratio of predators were analysed with logistic regression in Gen Stat, including the factors prey species and plant cultivar. T-probabilities were calculated to test pair-wise differences between means. Development time, adult dry weight, wing length, and head width were analysed using a three-way multivariate analysis of variance (MANOVA), including the factors prey species, plant cultivar and sex of the predator. MANOVA is used to test difference among groups for multiple dependant variables simultaneously. Besides this 'overall effect', MANOVA also provides results from the univariate analysis for each individual performance parameter.

Pupal weight was not included in the MANOVA, because sex of the pupae could not be determined, and was therefore analysed only by a two-way ANOVA on the factors prey species and plant cultivar. If necessary to obtain normally distributed data, log-transformation was applied. Significant differences amongst prey treatments were further analysed with a post-hoc Tukey test.

3.3 Results

3.3.1 Aphid performance

Nymphal survival of *M. persicae* was on average 63 ± 3% (mean ± SE) and did not differ among cultivars (logistic regression, *d.f.* = 3, deviance ratio = 1.39, $P = 0.256$). Development time differed among the cultivars (ANOVA, $F_{3,181} = 7.71$, $P < 0.001$). Nymphs on cultivar Lennox developed slower than on the other three cultivars (post-hoc LSD tests; development time on Lennox 13.0 ± 0.4 days [mean ± SE], on the other three cultivars on average 11.1 ± 0.2 days). Aphid multiplication factor increased over time (repeated measures ANOVA, $F_{6,336} = 337.20$, $P < 0.001$) and was different between the cultivars (repeated measures ANOVA, $F_{3,56} = 5.55$; $P = 0.002$). At the end of the experiment (day 34), the multiplication factor of *M. persicae* was more than twice as high on Christmas Drumhead and Badger Shipper (107 ± 30 and 100 ± 17 per plant respectively) than on Rivera and Lennox (39 ± 10 and 47 ± 9 per plant respectively).

Brevicoryne brassicae multiplication factors increased faster than *M. persicae* multiplication factors (repeated measures ANOVA, $F_{1,127} = 152.16$; $P < 0.001$) (Fig. 3.1). The ranking of the four white cabbage cultivars in terms of aphid multiplication factors of *B. brassicae* on these cultivars was similar to that of *M. persicae* (Fig. 3.1).

3.3.2 GLS

3.3.2.1 GLS in phloem of white cabbage plants

At both phloem sampling times, phloem samples contained higher concentrations of indole GLS than aliphatic GLS, as analysed for all four cultivars combined (Mann-Whitney U, first sampling: $U = 156.00$, $P < 0.001$; second sampling: $U = 309.00$, $P < 0.001$; Table 3.1). Aromatic GLS were not detected in the phloem. There was no difference in total GLS concentrations in phloem between both sampling times (Mann-Whitney U, $U = 551.00$, $P = 0.053$; Table 3.1).

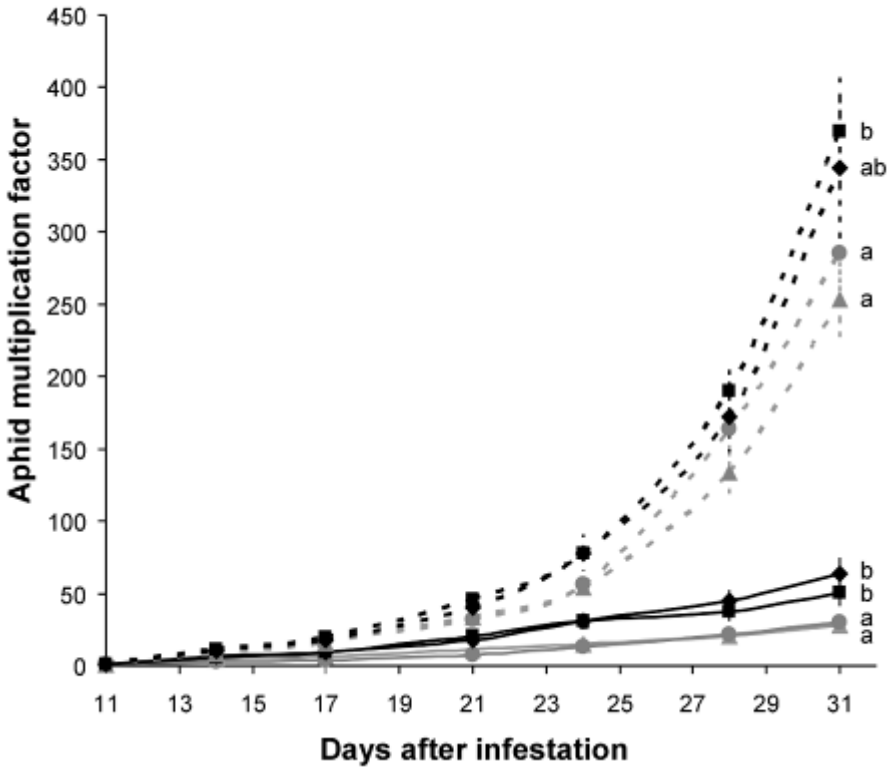


Fig. 3.1 *Multiplication factors (mean ± SE) of Myzus persicae (solid lines) and Brevicoryne brassicae (dotted lines) during 31 days of infestation of four white cabbage cultivars: Rivera (grey triangle), Lennox (grey circle), Christmas Drumhead (black square) and Badger Shipper (black diamond). Cultivar effects on multiplication factors were tested separately per aphid species, and different letters indicate differences between aphids reared on the different white cabbage cultivars at the level of $P < 0.05$ (post-hoc LSD tests). n is 15-18 per aphid species x cultivar combination. Data on B. brassicae were derived from Broekgaarden et al. (2008).*

There were no differences in concentrations of total, indole and aliphatic GLS in the phloem among the different cultivars (Kruskal Wallis H test, *d.f.* = 3, *n* = 10 per cultivar, $P > 0.05$ for all analyses), except for the second sampling time when the phloem of Badger Shipper plants contained higher total GLS concentrations than Lennox (Mann-Whitney U, $U = 11.00$, $P = 0.004$). GLS profiles in the phloem were not different among the cultivars (no significant PLS-DA components could be extracted).

Table 3.1 Mean (± SE) concentration (n = 40) of glucosinolate compounds detected in the phloem of white cabbage (averaged over four cultivars) at two sampling dates and in *Brevicoryne brassicae* nymphs and adults

Abbreviation	Trivial name	Scientific name	Phloem (1st)[a]	Phloem (2nd)[a]	L3 aphid[b]	Adult aphid[b]
Aliphatic GLS[c]						
GNA	Gluconapin	3-butenylGLS	328 ± 66	274 ± 56	1.81 ± 0.47	2.98 ± 0.68
IBE	Glucoiberin	3-methylsulfinylpropylGLS	n.d.[d]	n.d.	22.95 ± 2.73	35.44 ± 4.39
IBV	Glucoiberverin	3-methylthiopropylGLS	n.d.	n.d.	0.50 ± 0.12	n.d.
PRO	Progoitrin	2-OH-3-butenylGLS	n.d.	n.d.	8.85 ± 2.03	14.94 ± 2.74
RAPH	Glucoraphanin	4-methylsulfinylbutylGLS	n.d.	116 ± 29	15.89 ± 3.38	31.50 ± 7.48
SIN	Sinigrin	2-propenylGLS	n.d.	n.d.	n.d.	43.49 ± 5.31
Total aliphatic GLS			328 ± 66	390 ± 63	49.98 ± 6.63	128.34 ± 16.22
Indole GLS[c]						
GBC	Glucobrassicin	3-indolylmethylGLS	60 ± 13	72 ± 13	14.12 ± 1.24	11.16 ± 1.53
NEO	Neo-glucobrassicin	1-methoxy-3-indolylmethylGLS	54 ± 5	172 ± 33	n.d.	0.15 ± 0.03
4MeOH	4-methoxyglucobrassicin	4-methoxy-3-indolylmethylGLS	49 ± 12	105 ± 21	0.85 ± 0.07	0.61 ± 0.09
4OH	4-hydroxyglucobrassicin	4-OH-3-indolylmethylGLS	931 ± 88	345 ± 28	0.67 ± 0.09	0.74 ± 0.13
Total indole GLS			1089 ± 94	694 ± 63	15.64 ± 1.26	12.67 ± 1.60
Total GLS			1417 ± 111	1083 ± 87	65.62 ± 7.43	141.01 ± 17.35

[a] pmol g^{-1} dry weight leaf. [b] µmol g^{-1} dry weight aphids. [c] Glucosinolates (GLS) are grouped according to their biosynthetic origin. [d] n.d. = compound not detected

3.3.2.2 GLS in Brevicoryne brassicae

In third instar and adult aphids, concentrations of aliphatic GLS were higher than concentrations of indole GLS (Mann-Whitney U, L3 aphids: $U = 255.00$, $P < 0.001$; adult aphids: $U = 14.00$, $P < 0.001$; Table 3.1; Fig. 3.2). This contrasts to phloem samples, in which indole GLS concentrations were higher than those of aliphatic GLS. Aromatic GLS were not detected in *B. brassicae.* Adult aphids contained on average two times higher total concentrations of GLS than third instar nymphs when averaged over all cultivars (Mann-Whitney U, $U = 326.00$, $P < 0.001$; Table 3.1; Fig. 3.2), although adult aphids on Christmas Drumhead contained only about 70% more GLS than third instar nymphs (Fig. 3.2). Compared with third instar nymphs, adult aphids contained higher concentrations of aliphatic and lower concentrations of indole GLS when averaged over all cultivars (Mann-Whitney U, aliphatic: $U = 269.00$, $P < 0.001$; indole: $U = 520.00$, $P = 0.024$; Table 3.1; Fig. 3.2).

In both aphid developmental stages, there were no differences in concentrations of indole, aliphatic and total GLS among aphids reared on the different cultivars (Krukal Wallis H test, *d.f.* = 3, $P > 0.05$ for all analyses; Fig. 3.2). In contrast to the total GLS concentration, the GLS profiles of third instar nymphs did differ among nymphs reared on the different cultivars (3 PLS-DA principal components, $R_2X_{cum} = 0.811$, $R_2Y_{cum} = 0.363$, $Q_{2cum} = 0.201$; Fig. 3.3a). PLS-DA mostly separated GLS profiles of aphids reared on Christmas Drumhead and Badger Shipper from profiles of aphids reared on Rivera and Lennox (Fig. 3.3a). Fig. 3.3b shows the contribution of the GLS compounds to the discrimination among the aphid groups, based on the first two principal components. GLS profiles in adult aphids did not differ among aphids feeding on different cultivars (no significant PLS-DA components could be extracted).

3.3.2.3 Correlations between GLS in phloem and Brevicoryne brassicae

No correlations in indole, aliphatic and total GLS concentrations between the phloem of a plant and aphids feeding on that plant were found for both aphid developmental stages (Spearman's correlation, r_s values were between -0.039 and 0.216 and $P > 0.05$ for all correlations). Furthermore, we did not find a relationship between GLS profiles in the phloem and profiles in the aphids feeding on these plants at both sampling times (no significant PLS components could be extracted).

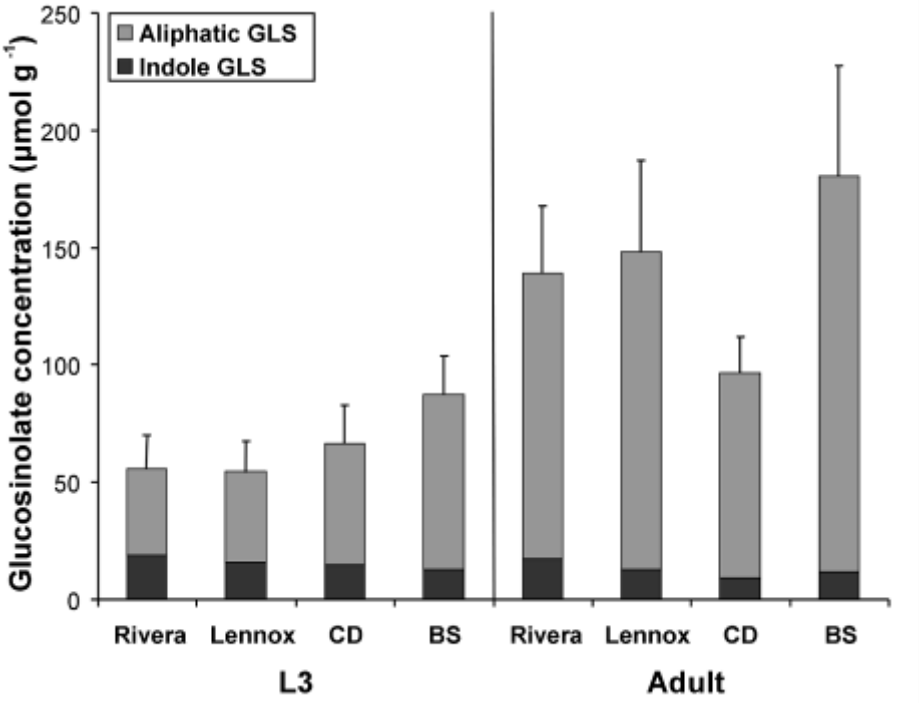


Fig. 3.2 *Total glucosinolate (GLS) concentration (mean + SE) in third instar nymphs (L3) and adult Brevicoryne brassicae when reared on four white cabbage cultivars: Rivera, Lennox, Christmas Drumhead (CD) and Badger Shipper (BS). GLS were, based on their biosynthetic origin, divided into indole and aliphatic GLS. n = 10 for all aphid stage x plant cultivar combinations and for each sample, several hundreds of aphids collected from one plant were pooled.*

3.3.3 Predator performance

3.3.3.1 Survival

Survival of *C. carnea* until adult emergence was on average 92%. Prey species, plant cultivar or the interaction between both did not affect survival of *C. carnea* (logistic regression, $P > 0.05$ for both factors and the interaction). Survival of *E. balteatus* until adult emergence was on average 60%. Survival of *E. balteatus* was affected by prey species (logistic regression, *d.f.* = 1, deviance ratio = 11.79, $P < 0.001$) as it was lower when fed *B. brassicae* (49% survival) than when fed *M. persicae* (70% survival). Plant cultivar or its interaction with prey species had no effect on *E. balteatus* survival (logistic regression, $P > 0.05$ for both analyses).

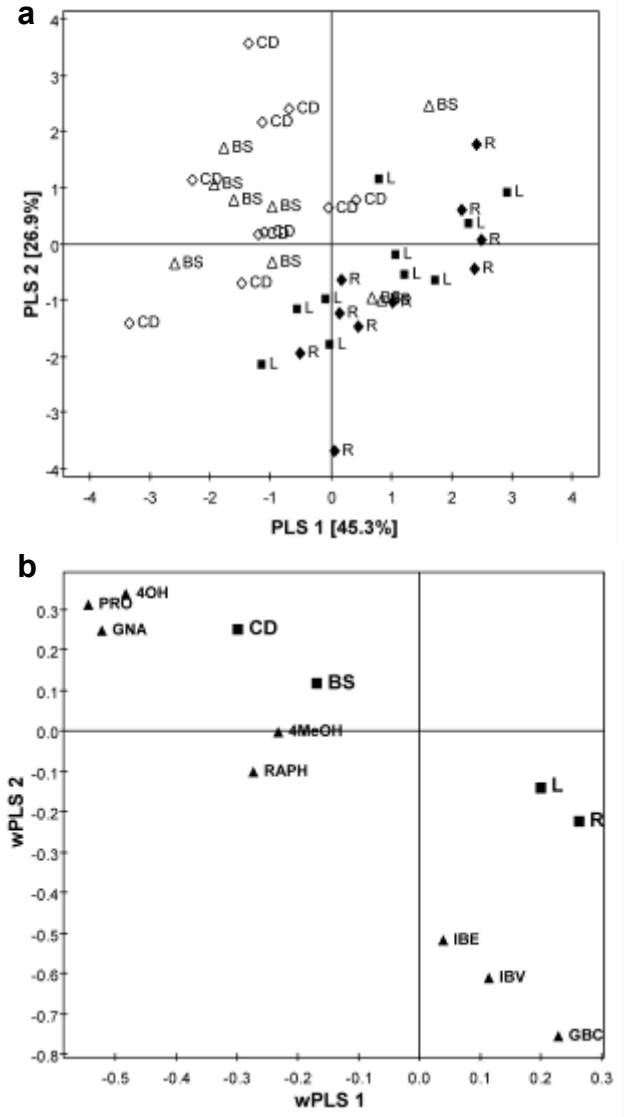


Fig. 3.3 *Multivariate data analysis of the glucosinolate (GLS) profiles of third instar Brevicoryne brassicae nymphs reared on four white cabbage cultivars by PLS-DA (projection to latent structures-discriminant analysis). Plant cultivars are Rivera (R), Lennox (L), Christmas Drumhead (CD) and Badger Shipper (BS).* ***a*** *Score plot of the first two components of PLS-DA, which shows the distinction in GLS profiles of B. brassicae nymphs reared on the different cultivars. In brackets the percentage of variation explained is indicated;* ***b*** *Loading plot of the first two components of PLS-DA, which shows the contribution of each of the GLS compounds to the discrimination between the B. brassicae groups reared on the four plant cultivars. Aliphatic GLS: GNA = gluconapin, IBE = glucoiberin, IBV = glucoiberverin, PRO = progoitrin, RAPH = glucoraphanin. Indole GLS: GBC = glucobrassicin, 4MeOH = 4-methoxyglucobrassicin, 4OH = 4-hydroxyglucobrassicin*

3.3.3.2 Development time, adult weight, and adult size

Prey species, plant cultivar, and predator sex affected the performance of both predator species in terms of development time and adult weight and size (Table 3.2). Although for *E. balteatus* interactions between the factors were observed (Table 3.2), effects on performance were mostly consistent for both predator species. In general, both predator species developed faster, although into smaller adults, when fed the generalist *M. persicae* compared with the specialist *B. brassicae* (Fig. 3.4; Fig. 3.5). For *C. carnea*, development was

Table 3.2 Effects of prey species, plant cultivar, predator sex, and their interactions on performance parameters of *Chrysoperla carnea* and *Episyrphus balteatus* as analysed by MANOVA

		Prey species (*d.f.* = 1)		**Plant cultivar (*d.f.* = 3)**		**Sex (*d.f.* = 1)**		**Prey*Cultivar (*d.f.* = 3)**		**Prey*Sex (*d.f.* = 1)**		**Cultivar*Sex (*d.f.* = 3)**		**Prey*Cultivar *Sex (*d.f.* = 3)**	
Param.	**Predator**	*F*[a]	*P*[b]	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Overall effect[c]	*C. carnea*	16.30	**<0.001**	5.97	**<0.001**	62.87	**<0.001**	1.58	0.093	0.54	0.708	0.95	0.499	1.21	0.270
	E. balteatus	68.63	**<0.001**	7.22	**<0.001**	25.73	**<0.001**	3.07	**<0.001**	3.80	**0.006**	1.93	**0.029**	1.08	0.372
Dev. time	*C. carnea*	31.76	**<0.001**	8.56	**<0.001**	9.44	**0.002**	2.00	0.115	0.0	0.981	1.25	0.292	0.22	0.882
	E. balteatus	243.24	**<0.001**	11.09	**<0.001**	2.74	0.100	6.60	**<0.001**	1.34	0.248	0.88	0.454	1.04	0.378
Pupal weight[d]	*C. carnea*	3.73	0.054	17.45	**<0.001**	n.m.[e]	n.m.	2.67	**0.048**	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
	E. balteatus	6.98	**0.009**	8.70	**<0.001**	n.m.	n.m.	2.71	**0.047**	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Adult weight	*C. carnea*	15.77	**<0.001**	18.05	**<0.001**	160.47	**<0.001**	2.11	0.100	0.45	0.503	0.92	0.431	0.97	0.407
	E. balteatus	22.57	**<0.001**	18.39	**<0.001**	25.68	**<0.001**	3.96	**0.010**	11.86	**0.001**	1.21	0.309	0.66	0.576
Head width	*C. carnea*	3.11	0.079	9.20	**<0.001**	49.39	**<0.001**	3.03	**0.030**	1.97	0.161	0.85	0.468	1.55	0.202
	E. balteatus	3.34	0.070	9.88	**<0.001**	51.73	**<0.001**	4.06	**0.008**	3.70	0.056	1.72	0.165	0.97	0.409
Wing length	*C. carnea*	1.27	0.261	8.56	**<0.001**	168.87	**<0.001**	0.80	0.496	0.15	0.695	0.48	0.699	4.45	**0.005**
	E. balteatus	1.93	0.167	7.88	**<0.001**	1.67	0.198	1.64	0.182	3.88	0.051	2.81	**0.042**	0.49	0.693

[a] F-values were rounded off to two decimals. [b] Bold printed numbers show significant effects at P < 0.05 as analysed by MANOVA. [c] Overall effect' is the effect on all performance parameters (Dev. time, Adult weight, Head width and Wing length) simultaneously. [d] Pupal weight was analysed only by two-way ANOVA and was not included in the MANOVA model, because the sex of the pupae could not be determined. [e] Not measured, because the sex of the pupae could not be determined.

fastest and adults were larger when fed aphids (both the specialist and the generalist aphid) reared on Christmas Drumhead and Badger Shipper, while development was slowest and adults were smaller when fed aphids reared on Rivera and Lennox (Fig. 3.4; Fig. 3.5). For *E. balteatus*, the effect of plant cultivar on development time and adult size was less consistent and depended on the prey species (Table 3.2), but mostly similar cultivar effects were observed as for *C. carnea* (Fig. 3.4; Fig. 3.5).

3.3.3.3 Effect of predator sex

Sex ratios were 48% females for *C. carnea* and 52% females for *E. balteatus* and did not differ among the different prey species x plant cultivar combinations (logistic regression, $P > 0.05$ for all combinations). The sex of the predatory larvae affected their performance (Table 3.2). Irrespective of prey species or host plant, *C. carnea* females developed slower than males (24.3 ± 0.2 days [mean ± SE] and 23.8 ± 0.2 days respectively) and developed into heavier adults than males (2.04 ± 0.02 mg and 1.72 ± 0.02 mg respectively). Development times of *E. balteatus* females and males (20.0 ± 0.3 days and 20.1 ± 0.3 days respectively) did not differ, but only when fed *B. brassicae*, *E. balteatus* females developed into lighter adults than males (3.20 ± 0.10 mg and 3.91 ± 0.15 mg respectively).

3.4 Discussion

3.4.1 Prey species effect

The two predator species, *C. carnea* and *E. balteatus*, exhibited slower development and *E. balteatus* exhibited lower survival when fed the specialist herbivore *B. brassicae*, than when fed the generalist herbivore *M. persicae*. Although the two aphid species probably differ in many ways, and displayed differential population growth on white cabbage, we propose that the observed difference in predator performance can be attributed to an important extent to the difference in concentrations of GLS and their hydrolysis products between the prey species (Gols and Harvey 2009). *Brevicoryne brassicae* sequesters GLS in high concentrations (100-150 times higher than in the phloem of its host plant, according to Hopkins et al. (2009)) and contains its own aphid-specific myrosinase that hydrolyses the GLS in its body upon damage by natural enemies (Jones et al. 2001; Francis et al. 2002). *Myzus persicae* does not sequester GLS, and excretes the ingested GLS in the honeydew (Francis et al. 2001b), but it does contain GLS in the gut that could potentially harm

predators. However, *M. persicae* does not contain myrosinases that could break down the GLS into toxic breakdown products. Therefore, predators face much higher concentrations of GLS and GLS hydrolysis products when feeding on *B. brassicae* than when feeding on *M. persicae*, which seems to correlate negatively with predator performance as observed in a separate experiment.

Interestingly, we document that the sequestration of GLS by *B. brassicae* from the host plant's phloem is selective. While indole GLS dominated in the phloem of white cabbage plants, aliphatic GLS dominated in the aphids. (Note that our method of collecting phloem sap inherently sampled a small amount of mesophyll fluids mixed with the phloem sap). Total GLS concentrations were more than two times higher in adult aphids than in third instar nymphs, confirming the observation by Kazana et al. (2007) that *B. brassicae* continues to sequester GLS during its development. Furthermore, from the third instar to the adult stage, aliphatic GLS were sequestered in higher concentrations, while concentrations of indole GLS decreased. The high sequestration of aliphatic GLS, but not of indole GLS, can be explained by the difference in toxicity between both. Formation of toxic hydrolysis products of plant aliphatic GLS is prevented in aphids due to the intercellular

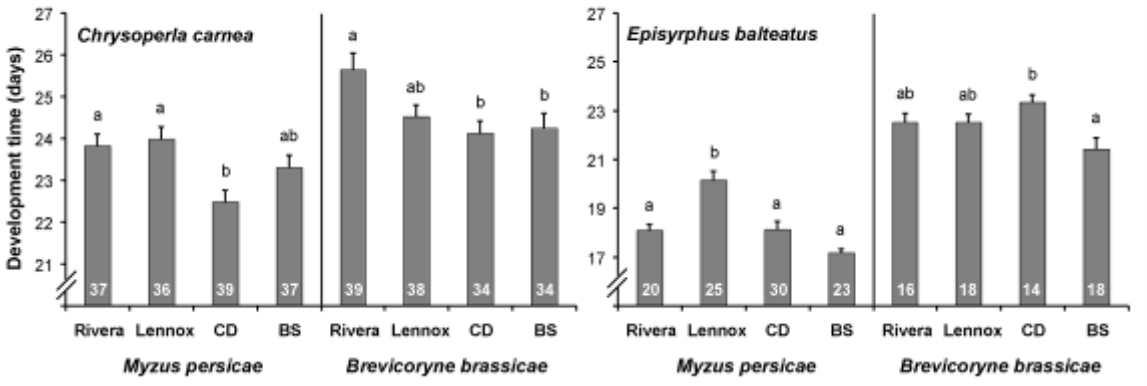


Fig. 3.4 *Development time in days (mean + SE) of Chrysoperla carnea and Episyrphus balteatus when fed Myzus persicae or Brevicoryne brassicae reared on one of four different white cabbage cultivars: Rivera, Lennox, Christmas Drumhead (CD) and Badger Shipper (BS). Within a prey species, different letters above the bars indicate significant differences related to prey being reared on different cultivars at the level of P < 0.05 (Tukey post hoc multiple comparison test). n tested per prey x cultivar combination is indicated in each bar*

path taken by aphid stylets to reach the phloem (Tjallingii and Hogen Esch 1993), thus allowing aphids to ingest phloem GLS without bringing these compounds into contact with plant myrosinases (Andreasson et al. 2001; de Vos et al. 2007; Kim and Jander 2007). Indole GLS, in contrast, have been shown to be broken down to toxic hydrolysis products independently of myrosinase activity (Kim and Jander 2007; Kim et al. 2008). Indole GLS may, therefore, be detrimental for *B. brassicae*, as was suggested by Cole (1997), providing an explanation for the low sequestration of these GLS. Aliphatic GLS undergo fast enzymatic degradation by purified aphid myrosinase, whereas the lowest activity of aphid-produced myrosinase is observed with indole GLS (Francis et al. 2002), and higher sequestration of aliphatic GLS by *B. brassicae* may therefore lead to higher toxicity to predators, without affecting aphid performance itself.

The mechanism underlying the high sequestration of aliphatic GLS, but low sequestration of indole GLS by *B. brassicae* could be that transporters for GLS uptake in insects may be rather specific. While the GLS-sequestering sawfly *Athalia rosae* sequesters mostly aliphatic GLS, and almost no indole GLS (Müller 2009), another species of this genus, *A. liberta*, has been shown to be able to sequester indole GLS (Opitz et al. 2010). Unfortunately, nothing

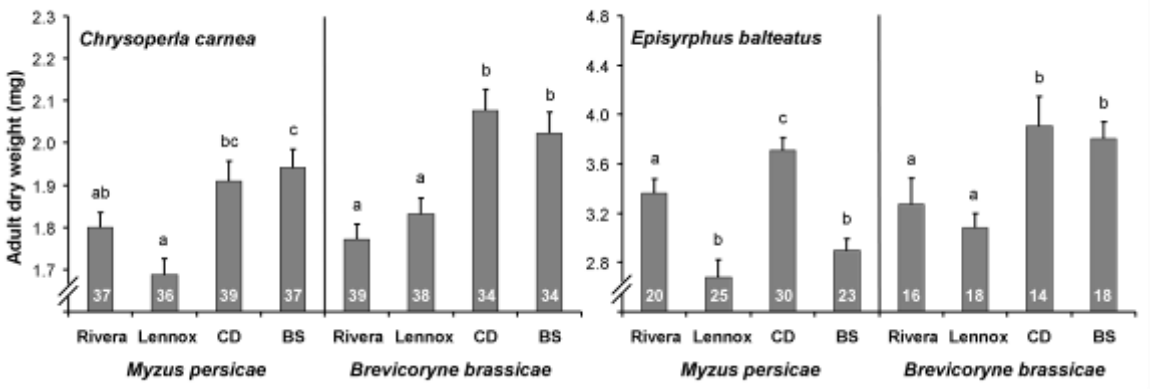


Fig. 3.5 *Adult dry weight in milligrams (mean + SE) of Chrysoperla carnea and Episyrphus balteatus when fed Myzus persicae or Brevicoryne brassicae reared on one of four different white cabbage cultivars: Rivera, Lennox, Christmas Drumhead (CD) and Badger Shipper (BS). Within a prey species, different letters above the bars indicate significant differences related to prey being reared on different cultivars at the level of P < 0.05 (Tukey post hoc multiple comparison test). n tested per prey x cultivar combination is indicated in each bar*

is known yet about the exact mechanisms underlying GLS sequestration or the specificity of GLS transporters (Opitz et al. 2010).

Although development of both predator species was slower and survival of *E. balteatus* was lower when fed *B. brassicae*, predator size was larger when feeding on this aphid species. Predator size is generally positively correlated with lifetime fecundity, as was shown for *E. balteatus* (Branquart and Hemptinne 2000). Fitness of an individual, however, is not necessarily affected equally by the different performance parameters we have measured. Aphid species are so-called *r*-selected, colonizing species, characterised by fast development, fluctuating population densities in response to changing environments and abundant offspring production (Lewontin 1965; Caswell and Hastings 1980; Carter and Dixon 1981; Ankersmit et al. 1986). Under such selection pressures an increase in developmental rate has a more pronounced effect on an individual's fitness than an increase in lifetime fecundity (Caswell and Hastings 1980). If natural enemies of aphids are under the same selection pressures as their prey, short development time would also be favoured over offspring production, as has been suggested for aphid parasitoids (Sequeira and Mackauer 1994 and references therein). We assume, therefore, that development time and survival of aphid predators are more important components of their fitness than adult size.

3.4.2 Plant cultivar effect

The performance of a natural enemy is often positively correlated with the performance of its host or prey (Benrey et al. 1998; Sznajder and Harvey 2003). In support of this, the performance of *C. carnea* and *E. balteatus* when fed aphids reared on the four cultivars reflected the performance of the aphids themselves on these cultivars: aphids and predators performed better on Christmas Drumhead and Badger Shipper than on Rivera and Lennox (Fig. 3.1, 3.4 and 3.5). However, in the case of *B. brassicae*, not only aphid performance itself, but also the GLS content of the aphids has likely influenced predator performance, because GLS concentrations reached high levels in these aphids. These high concentrations of GLS likely led to high concentrations of GLS hydrolysis products after breakdown by the aphid myrosinase, although we did not quantify GLS and their hydrolysis products separately. Predators developed generally faster and into larger adults when fed *B. brassicae* reared on Christmas Drumhead and Badger Shipper than when fed *B. brassicae* reared on Rivera and Lennox. These two distinct

groupings in predator performance matched the groupings of GLS profiles of the aphids reared on the different plant cultivars, but not the total indole, total aliphatic or overall total GLS concentrations. Although we believe that the differences in GLS profiles, rather than total concentrations of GLS, among aphids developing on the different cultivars influenced predator performance, we cannot rule out potential effects of other aspects of aphid quality on predator performance. In a study with *A. rosae*, haemolymph of the larvae deterred ants and predatory wasps more strongly than the individual major GLS compounds in the haemolymph (Müller et al. 2002; Müller and Brakefield 2003). It is, however, not clear whether this stronger deterrence was due to other GLS compounds in the haemolymph, in agreement with our hypothesis that GLS profiles rather than total concentrations influence natural enemies, or whether this stronger deterrence was due to completely different compounds.

3.4.3 Conclusion

Our study shows that not only the prey species, but also the plant cultivar can have an effect on the performance of predators. When fed *B. brassicae* populations reared on different plant cultivars, differences in predator performance matched differences in GLS profiles among the aphids, although we did not test other aspects of aphid chemistry. Predator performance, in terms of survival and development time, was lower when predators were fed the specialist aphid *B. brassicae* that selectively sequestered GLS from its host plant and contains its own aphid-specific myrosinase, than when fed the non-sequestering *M. persicae*. Breakdown products of GLS are known to confer direct resistance against a wide variety of herbivores, and our results imply that aphid GLS and their hydrolysis products negatively affected aphid predators. This suggests that in the tritrophic system tested, in which GLS are the main secondary metabolites, there might be a conflict between direct and indirect resistance.

The conflict between direct and indirect resistance may not be a general trend in nature and may, for example, not arise for specialist natural enemies. Specialist natural enemies that are adapted to feeding on GLS-containing herbivores, such as the aphid parasitoid *Diaeretiella rapae*, are probably not affected negatively by higher concentrations of GLS in their hosts (Le Guigo et al. 2011). Parasitoid wasps such as *D. rapae* have been shown to be attracted to volatile breakdown products of GLS that are indicators of host presence (Bradburne and Mithen 2000; Blande et al. 2007). For attraction of

natural enemies by volatile breakdown products of GLS, not only total concentrations of GLS are important, but also the side-chain of the GLS, as breakdown of aliphatic GLS leads to higher emission of volatiles than breakdown of indole GLS (Hopkins et al. 2009).

Our findings suggest that plants might be subject to a conflict between the consequences of producing higher or lower levels of GLS. Higher levels enhance resistance against herbivores and attract specialist natural enemies; lower levels improve the performance of generalist natural enemies, possibly leading to balancing selection on plant GLS levels. This hypothesis may be tested by varying only levels and profiles of GLS hydrolysis products in the diet of natural enemies, either by using artificial diets containing GLS that are offered in combination with myrosinases, or by rearing host and prey on plants that have been genetically engineered to produce modified GLS levels and profiles.

3.5 Acknowledgements

We thank two anonymous reviewers for constructive comments on an earlier version of the manuscript; Colette Broekgaarden for the permission to use *B. brassicae* performance data; Rieta Gols, Roland Mumm, and Erik Poelman for help with the statistics; Koppert Biological Systems for the delivery of *E. balteatus* pupae and *C. carnea* eggs; CGN and Bejo Zaden for providing seeds of the cabbage cultivars and Unifarm, especially Andre Maassen and Alex Super, for plant rearing. This work was supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (number 838.06.010).

4

Relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance on *Brassica oleracea*

Martine Kos, Colette Broekgaarden, Patrick Kabouw, Kirsten Oude Lenferink, Erik H. Poelman, Louise E.M. Vet, Marcel Dicke and Joop J.A. van Loon

Published in *Functional Ecology* 25: 1113-1124 (2011)

Abstract

Arthropod communities are structured by complex interactions between bottom -up (resource-based) and top-down (natural enemy-based) forces. Their relative importance in shaping arthropod communities, however, continues to be under debate. Bottom-up and top-down forces can be affected by intraspecific plant variation, for example by differences in concentrations of secondary metabolites that affect herbivore abundance through plant quality (bottom-up) or attract natural enemies of these herbivores (top-down). Our objective was to investigate whether herbivore abundance is more strongly affected by plant-mediated bottom-up or top-down forces.

We used a model system of four cultivars of *Brassica oleracea* that show a high degree of variation in several plant traits, resistance to herbivores and attraction of natural enemies. During two field seasons, we recorded the abundance of several herbivorous and carnivorous insect species. To assess the relative importance of bottom-up and top-down forces, we quantified chemical and morphological traits of the cultivars (bottom-up) and assessed parasitisation of herbivores and predator oviposition on plants inoculated with a controlled number of herbivores (top-down).

We show that intraspecific variation in plant chemistry and morphology consistently affects the abundance of insect herbivores and their natural enemies, resulting in cascading effects on tritrophic interactions in the associated insect community. Foliar profiles of glucosinolates and leaf toughness appeared most important for these effects. *Brassica oleracea* cultivars that harboured the largest numbers of herbivores also harboured the largest numbers of natural enemies. Differences in the fraction of herbivores parasitized and in predator oviposition on plants inoculated with a controlled number of herbivores could not explain the differences in natural abundance of herbivores.

Although abundance of herbivores is most likely influenced by a combination of bottom-up and top-down forces, it appears that in the tritrophic system investigated, bottom-up forces (plant chemistry and morphology) were more important for herbivore abundance than plant-mediated top-down forces (attraction and arrestment of natural enemies).

4.1 Introduction

Arthropod communities are structured by bottom-up (resource-based) and top-down (natural enemy-based) forces (Hunter and Price 1992; Forkner and Hunter 2000; Aquilino et al. 2005). Effects of plants on herbivores can 'cascade up' the food web and determine species diversity and population dynamics at higher trophic levels (Hunter and Price 1992; Bukovinszky et al. 2008). Carnivores can have effects on herbivore communities via predator-prey interactions and may also, indirectly, influence the abundance of plant species (Schmitz et al. 2000; Halaj and Wise 2001). Although often studied separately, bottom-up and top-down forces do not act in isolation but interact in complex ways (Dicke and Hilker 2003). Their relative importance in shaping arthropod communities, however, continues to be under debate (Hunter and Price 1992; Forkner and Hunter 2000; Schmitz et al. 2000; Walker et al. 2008).

Bottom-up and top-down forces can both be affected by intraspecific plant variation. For example, differences in concentrations of secondary metabolites among wild plant populations or cultivars can affect the abundance of insect herbivores and consequently, the abundance of their natural enemies (bottom-up forces) (Whitham et al. 2003; Bukovinszky et al. 2008; Newton et al. 2009b; Poelman et al. 2009b). Intraspecific differences in quality or quantity of volatile blends can result in differential attraction of natural enemies of herbivores (Hoballah et al. 2002; Gols et al. 2009; Poelman et al. 2009a), leading to plant-mediated differences in top-down control of herbivores. Thus, intraspecific variation can have large effects on insect communities via both bottom-up and top-down mechanisms (Crutsinger et al. 2006; Johnson et al. 2006; Newton et al. 2009b; Poelman et al. 2009b).

Here, we investigated whether herbivore abundance is more strongly affected by plant-mediated bottom-up or top-down forces. We used a model system of four *Brassica oleracea* L. cultivars that differ in their resistance to herbivores and interactions with natural enemies, and exhibit significant intraspecific variation in glucosinolates (GLS) and herbivore-induced volatiles (Broekgaarden et al. 2008; Poelman et al. 2008b; Poelman et al. 2009a; Kabouw et al. 2010a). GLS are defensive secondary plant metabolites characteristic for Brassicaceae and hydrolysed upon disruption of plant tissue by the enzyme myrosinase and, depending on the type of GLS, form hydrolysis products such as (iso)thiocyanates and nitriles that are toxic to (mainly generalist) herbivores (Bones and Rossiter 2006; Halkier and Gershenzon 2006). We quantified the abundance of several insect herbivores

and their natural enemies on each of the *B. oleracea* cultivars during two field seasons. We assessed the importance of bottom-up forces on herbivore abundance by quantifying chemical and morphological traits of the cultivars, and we assessed the importance of top-down forces on herbivore abundance by quantifying parasitisation of herbivores and predator oviposition on each of the cultivars.

4.2 Materials and methods

4.2.1 General approach

A single common garden was established in an agricultural field near Wageningen, The Netherlands. In this common garden, we established 32 plots containing a monoculture of one of the four *B. oleracea* cultivars, resulting in eight plots per cultivar. In the common garden, we performed several experiments.

To quantify the abundance of several herbivores and carnivores over time, we monitored the nine central plants of each plot. We selected two herbivorous insect species that represent common members of the insect community on *B. oleracea* (Poelman et al. 2009b). In addition, we studied the abundance of the associated natural enemies. We repeated this experiment over two field seasons (2008 and 2009) to test for consistency of our results over time.

To investigate whether bottom-up forces explained the observed differences in herbivore abundance, we quantified chemical and morphological traits of the cultivars in the field. We used the same central plants of each plot as we used for quantifying insect abundance.

To investigate whether top-down forces explained the observed differences in herbivore abundance, we assessed parasitisation of herbivores and predator oviposition on plants inoculated with a controlled number of herbivores. We selected plants in each plot other than the nine central plants. We used a fixed number of herbivores to rule out host-density-dependent effects on top-down control of herbivores and to focus only on differences in attraction and arrestment of natural enemies among cultivars.

By combining the results from all experiments, the relative importance of bottom-up and top-down forces on herbivore abundance was assessed.

4.2.2 Plants and insects

Four white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba*) cultivars

were used: Christmas Drumhead and Badger Shipper (Centre for Genetic Resources, CGN, Wageningen, The Netherlands), representing older, open pollinated, cultivars, and Lennox and Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands), representing more recently cultivated, commercially grown, F1 hybrids. Seeds were germinated on peat soil (Lentse potgrond no. 1, Lent, The Netherlands) in a greenhouse at 20° ± 2°C; 40-70% relative humidity (RH) and a 16:8 h light:dark (L:D) photoregime. Plants were watered daily and received additional nutrients once a week [concentration 1.8 mg L^{-1} (N-P-K-MgO; 19-6-20-3); Kristalon Blauw, Hydro Agri, Rotterdam, The Netherlands]. Minimum light intensity was maintained above 210 PAR (µmol m^{-2} s^{-1}) by high-pressure sodium lamps (SON-T, Philips, Eindhoven, The Netherlands).

For the inoculation treatments in the field we used cultures of the leaf chewing *Plutella xylostella* L. (diamondback moth; Lepidoptera: Yponomeutidae) and the phloem sucking *Brevicoryne brassicae* L. (cabbage aphid; Hemiptera: Aphididae) (Fig. 4.1) that were maintained on Brussels Sprouts (*B. oleracea* L. var. *gemmifera* cv. Cyrus) in a climatised room at 22° ± 2°C, 60-70% RH and a 16:8 h L:D photoregime.

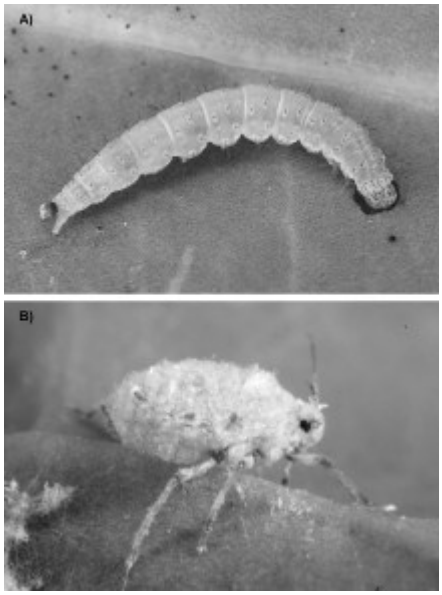


Fig. 4.1 *Caterpillar of the diamondback moth, Plutella xylostella (A) and nymph of the cabbage aphid, Brevicoryne brassicae (B) (photographs by Tibor Bukovinszky; www.bugsinthepicture.com).*

4.2.3 Common garden set-up

Ten days after germination, individual seedlings were transferred to peat soil cubes. Two-and-a-half-week old plants were moved outside the greenhouse to acclimatize to field conditions. In week 18 (late April) five-week-old plants were transplanted, with their soil cubes, into the soil at the common garden. We established 32 plots (6 x 6 m), each containing a monoculture of 49 plants of one of the four cultivars. Cultivars were assigned to plots using a randomized design. The plants were planted in a 7 x 7 square and separated by 75 cm of bare soil. A 6-m strip sown with *Lolium* and *Poa* grasses separated the plots. The common garden was fertilized once at the beginning of the season with dry pellets of organic fertilizer (Culterra, Workum, The Netherlands, N-P-K; 10-4-6 micro).

4.2.4 Insect abundance

From week 23 (early June) until week 36 (early September) in both study years, the nine central plants of each plot were monitored weekly for the presence of the following insect species: non-mining caterpillars and pupae of *P. xylostella* and pupae of its parasitoid *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae); colony size of *B. brassicae* aphids and other aphids; mummies (pupae of the parasitoid inside the host integument) of the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae); larvae and pupae of the predator *Episyrphus balteatus* de Geer (Diptera: Syrphidae) and eggs, larvae and pupae of the predator *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). For each week, we averaged the number of each of the selected insect species per plot.

4.2.5 Chemical and morphological traits of field plants

In 2008, we quantified GLS, amino acid, and sugar contents of intermediate-aged leaves of the nine central plants of each plot. In 2009, we adapted our method based on the results from 2008. Because sugar and amino acid contents of plants in 2008 were not correlated with insect abundance (see *Results*) we assessed only GLS composition in 2009. To test for differences in chemical composition among leaf ages, we analysed young (3rd youngest leaf), intermediate-aged (between 8th and 10th youngest leaf) and old (3rd oldest leaf) leaves separately in 2009. Each plant had ca. 20-25 leaves in total. Furthermore, we included measurements of morphological traits as we observed that the cultivars differed greatly in morphology.

4.2.5.1 Chemical traits (2008 and 2009)

Leaf material of four plants of each plot was sampled at the peak of insect abundance (week 29; mid July) in both years and pooled into one sample per plot. All plant material was flash-frozen in liquid nitrogen, transferred to a -80 ° C freezer, freeze-dried and ground into a fine powder of which approximately 100 mg was used in the analysis. GLS analyses were further performed as described by Kabouw et al. (2010a) and van Dam and Oomen (2008). Soluble sugars and amino acids were extracted and analysed as described previously by van Dam and Oomen (2008).

4.2.5.2 Morphological traits (2009)

In 2009, an index of the developmental stage of the nine central plants of the plots, on which insect abundance was measured, was recorded weekly according to de Moel et al. (1996). The relevant part of this scale ranges from 2.0 (first leaf appearance), through 3.0 (onset of head formation) and 4.0 (optimal harvest stage) to 4.9 (overripe and cracked).

In week 29 (mid July), four of the nine central plants were harvested for measuring morphological traits. We measured fresh weight of the plants by weighing the shoot. For analysis of the amount of leaf surface wax of each of the cultivars, a leaf disk with a diameter of 9.2 cm (66.48 cm^2) was cut from an intermediate-aged leaf from three plants per plot. All three leaf disks were dipped in 50 ml chloroform (99%, BDH laboratory supplies, England) for 30 seconds to collect leaf surface wax. The chloroform solution was evaporated to a volume of 1 ml by gently leading a nitrogen flow over the solution while heating it to 40°C. Fifty µl of the 1 ml sample was pipetted onto a pre-weighed glass cover slip (24 x 40 mm). After the chloroform had evaporated, the amount of wax was determined by re-weighing the cover slip. We averaged the amount of wax for the three sampled plants in each plot.

Leaf toughness was measured separately for young, intermediate-aged and old leaves of four plants of each plot. We determined the force that is required to penetrate the leaf as an indicator of leaf toughness using the Instron hardness meter (Instron 4301, Instron Int. Ltd). A pin with a diameter of 3.18 mm that generates a maximum force of 5 kN was used to penetrate the adaxial side of the leaf, in the centre of the leaf, 2 cm from the main vein, avoiding penetration of other veins.

Light reflectance by the leaf surface of young, intermediate-aged and old leaves of two plants per plot was measured by using a Perkin Elmer

spectrophotometer (Perkin Elmer, Lambda 950, UV/VIS Spectrometer, Shelton, Connecticut, USA). Measurements were made on the adaxial side of the leaf, in the top right corner, avoiding veins. Analyses were done on the percentage reflectance at 330 nm (representing relative reflectance of UV light), 550 nm (representing relative reflectance of green light) and 680 nm [representing relative reflectance of photosynthetically active wavelengths; Holmes and Keiller (2002)].

4.2.6 Parasitisation and predator oviposition responses to inoculated herbivores

We assessed parasitisation of *P. xylostella* caterpillars and *B. brassicae* aphids on plants inoculated with a controlled number of herbivores. A single plant of every plot was inoculated weekly from week 25 (mid June) until week 36 (early September). Every week we used a different plant in the plot, but the nine central plants and the outer rows of each plot were excluded.

4.2.6.1 Parasitisation of P. xylostella

Plants were inoculated with *P. xylostella* in the odd weeks of 2008 and weekly in 2009. Twenty second-instar caterpillars were placed on one leaf of the plant, recollected after three days, and reared on Brussels sprouts leaves until adult moth or adult parasitoid emergence. The sex of the wasps was recorded. Due to a problem with the *P. xylostella* rearing that started in August 2009, parasitisation of *P. xylostella* in 2009 was assessed only until week 30 (late July).

4.2.6.2 Parasitisation of B. brassicae *and predator oviposition*

Plants were inoculated with *B. brassicae* in the even weeks of 2008. Twenty adult aphids in clip cages were transferred to four leaves of each plant. Adult aphids and clip cages were removed after three days, and the offspring produced was standardized to 30 nymphs per plant. After four days, the nymphs were recollected from the field, fed with Brussels sprouts leaves and reared until mummy formation.

However, we adapted our method for *B. brassicae* aphids in 2009, because we observed that with increasing age of the plants, the mortality of aphids transferred to two of the cultivars (Rivera and Lennox) increased dramatically (data not shown). To assure survival of aphids on the plants, we used greenhouse-grown plants. Inoculation was done on a weekly basis from

week 23 (early June) until week 36 (early September). At the same time, oviposition by aphid predators could be tested by counting the number of eggs on the plants. To obtain potted plants, individual seedlings were transferred to 1.45 L pots eight days after germination and used when they were seven weeks old. Thirty-six adult aphids in clip cages were transferred to three young leaves of 15 plants of each *B. oleracea* cultivar. Adult aphids and clip cages were removed after three days, and the offspring produced was standardized to 90 nymphs per plant. The plants were transferred to the common garden and randomly distributed over 20 newly developed plots in sets of three plants of the same cultivar. These newly developed plots were bare plots of 1 m x 1 m established in the middle of the grass strip in between the previously described plots. After three days, the number of recovered aphids, *E. balteatus* eggs and *C. carnea* eggs was recorded. To check for parasitism by *D. rapae*, the recovered aphids were fed with Brussels sprouts leaves and reared until mummy formation. Per plot, we averaged the data of the three plants, resulting in five replicates per *B. oleracea* cultivar per week.

4.2.7 Statistical analysis

4.2.7.1 Insect abundance

Statistical analyses were performed using SPSS for Windows (15th edition, Chicago, Illinois, USA), unless indicated otherwise. Abundance over time of the selected insect species was analysed for both years separately using structured repeated measurements mixed models with the repeated structure type AR (1). The average number of the selected insect species per plant in a plot was log- or square-root normalized (based on which transformation provided the best model fit), and modelled by the factors cultivar, week (23-36), and the factorial interaction. Plot was used as a random factor. Mean differences between cultivars were compared using post-hoc LSD tests. Larvae and pupae of *C. carnea* were only observed occasionally and were omitted from the analysis.

We also tested differences in the fraction of naturally occurring herbivores that were parasitized using Generalized Linear Mixed Models (GLMMs) in the program GenStat (12th edition, VSN International, UK). Plot was included as a random factor. The study weeks that were included in the model were determined per model: weeks in which half of the plots did not contain any of the relevant herbivores were omitted from the analysis. In all GLMMs and GLMs (Generalized Linear Models) performed in this study, a

binomially distributed dependent variable with fixed binomial totals was used in the model (binomial distribution, logit link function, dispersion estimated). The factors cultivar, study week, and the interaction were included in a full-factorial design. See Table S4.1 in Supporting Information for a detailed description of each GLMM analysis.

4.2.7.2 Chemical and morphological traits of field plants

We used two different statistical approaches to assess the importance of chemical and morphological traits on insect abundance. First, we analysed differences in plant traits among the cultivars, to test whether there was intraspecific chemical and morphological variation in *B. oleracea*, and thereby potential for differences in bottom-up control of herbivores in this system. Second, to correlate plant traits with insect abundance, we performed multivariate regression analyses, in which we could assess the importance of the different chemical and morphological plant traits for insect abundance.

Differences in total concentrations of primary and secondary metabolites among *B. oleracea* cultivars were analysed by analysis of variance (ANOVA) or, if assumptions on normality were violated, Kruskal Wallis H-tests. For all ANOVAs performed in this study, post-hoc Tukey tests were used for pairwise differences among means. Similarly, for all Kruskal Wallis H-tests, Mann-Whitney U-tests were used for pairwise differences. For GLS, concentrations of indole and aliphatic GLS, were analysed separately. Because the only aromatic GLS (gluconasturtiin) was found in only trace amounts, this compound was excluded from statistical analysis. An unidentified sugar was found that contributed max. 6% of the total sugar content; this sugar was not included in the analysis.

Changes in developmental stage index of plants over time was analysed as described above for the insect abundance. The average fresh plant weight per plot was analysed by one-way ANOVA on the factor cultivar. The amount of wax on the surface of intermediate-aged leaves was analysed by Kruskal-Wallis H-tests. The force necessary to penetrate the leaf was averaged per plot, log-normalized and analysed by two-way ANOVA for the factors cultivar, leaf age, and the factorial interaction. Percentage of light reflectance at 330 nm, 550 nm and 670 nm was averaged per plot, arcsin-square root transformed and analysed by two-way ANOVA.

Metabolite profiles of intermediate-aged leaves were analysed by projection to latent structures-discriminant analysis (PLS-DA) in SIMCA-P (12th

edition, Umetrics, Umeå, Sweden) (Eriksson et al. 2006). PLS-DA is a multivariate discriminant analysis that we used to test whether GLS, amino acid and sugar profiles differed among cultivars.

The relationship between chemical and morphological traits of *B. oleracea* plants and insect abundance on those plants was analysed by partial least squares projections to latent structures (PLS) in SIMCA-P, an approach similar to regression analysis (Eriksson et al. 2006). For both years and for the different plant traits we constructed separate models. For each model, the variable importance in the projection (VIP) was calculated. Variables with a VIP value higher than 1 are most influential for the model (Eriksson et al. 2006). To pre-process data for PLS-DA and PLS analysis, they were log-normalized, mean-centred, and scaled to unit variance.

4.2.7.3 Parasitisation and predator oviposition responses to inoculated herbivores

The fraction of experimentally released *P. xylostella* caterpillars or *B. brassicae* aphids that were recovered from the field, the fraction of recollected caterpillars or aphids that were parasitized and the fraction of *D. semiclausum* adults that were female in the parasitisation and predator oviposition experiment were analysed by Generalized Linear Models in GenStat (see Table S4.1 for a detailed description of each GLM analysis). Two-sided t-probabilities were calculated to test pair-wise differences between means. The number of *E. balteatus* and *C. carnea* eggs were analysed by Kruskal-Wallis H -tests because these data were not normally distributed, and modelled by the factor cultivar.

4.3 Results

4.3.1 Insect abundance

In both years, all insect species showed clear population fluctuations over time (time effect, repeated measures mixed models, $P < 0.001$ for all insect species; Figs 4.2 and 4.3; Table S4.2). Despite these clear temporal effects, plant cultivar consistently affected insect abundance (cultivar effect, repeated measures mixed models, $P < 0.001$ for all insect species in either one or both years; Table S4.2). Overall, herbivores and their natural enemies were more abundant on Badger Shipper and Christmas Drumhead compared to Rivera and Lennox [Figs 4.2, 4.3 and S4.1; Table S4.2. Note that Figs 4.2 and 4.3 show the data of the study year in which the abundance of the herbivore was

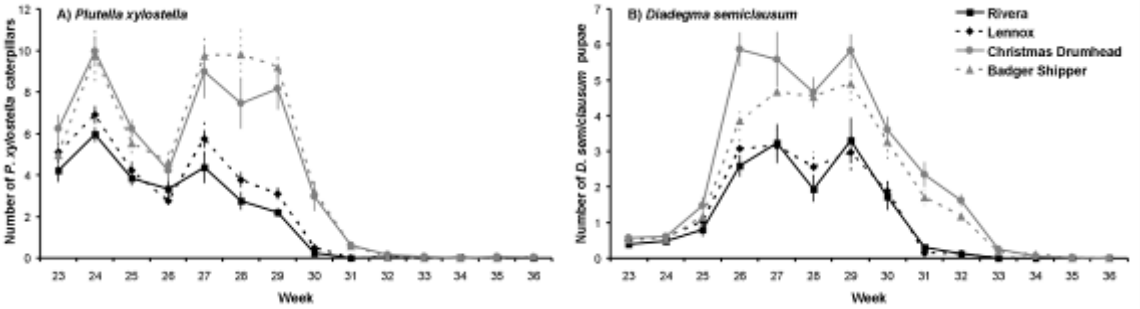


Fig. 4.2 *Abundance of Plutella xylostella caterpillars and their parasitoids over time on plants of four Brassica oleracea cultivars in a common garden experiment in 2009. (A) the lepidopteran herbivore P. xylostella (mean number of caterpillars per plant ± SE); (B) the parasitoid Diadegma semiclausum (mean number of pupae per plant ± SE).*

highest (2009 for *P. xylostella* and 2008 for *B. brassicae*) and Fig. S4.1 shows the data of the other study year].

The fractions of herbivores that were parasitized also changed over time in both years (time effect, GLMM, $P < 0.001$ for both herbivores; Table S4.3). For every herbivore-parasitoid complex, the fraction of herbivores that was parasitized was often smallest on cultivars that harboured the largest herbivore numbers (Christmas Drumhead and Badger Shipper) (Table S4.4), although differences among cultivars were mostly not significant (Table S4.3).

4.3.2 Chemical and morphological traits of field plants

4.3.2.1 GLS

GLS profiles were comparable between both study years, and were different among the *B. oleracea* cultivars (cultivar effect, 2008: 4 PLS-DA principal components, $R_2X_{cum} = 0.959$, $R_2Y_{cum} = 0.621$, $Q_{2cum} = 0.431$; 2009: 3 PLS-DA principal components, $R_2X_{cum} = 0.787$, $R_2Y_{cum} = 0.536$, $Q_{2cum} = 0.405$). The PLS-DA models showed that Rivera and Lennox were similar in terms of GLS profile, while Christmas Drumhead and Badger Shipper were different from each other and from Rivera and Lennox (Figs 4.4A and 4.4B). In both years, Christmas Drumhead had the highest and Badger Shipper the lowest concentrations of most GLS. Rivera and Lennox had the highest glucoiberin and sinigrin concentrations (Figs 4.4C and 4.4D).

In general, young leaves contained the highest and old leaves the lowest concentrations of indole, aliphatic and total GLS although patterns among cultivars differed to some extent (Table 4.1 and S4.5).

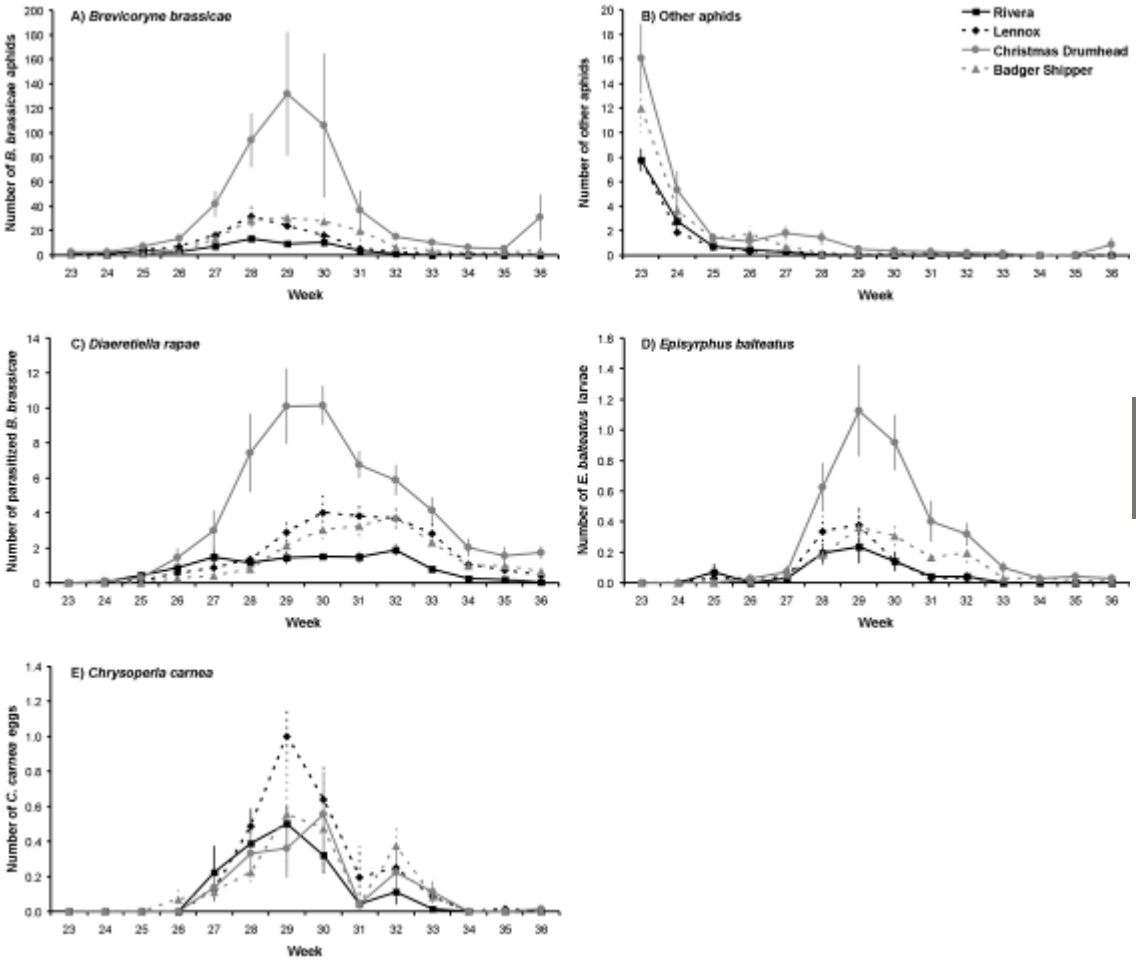
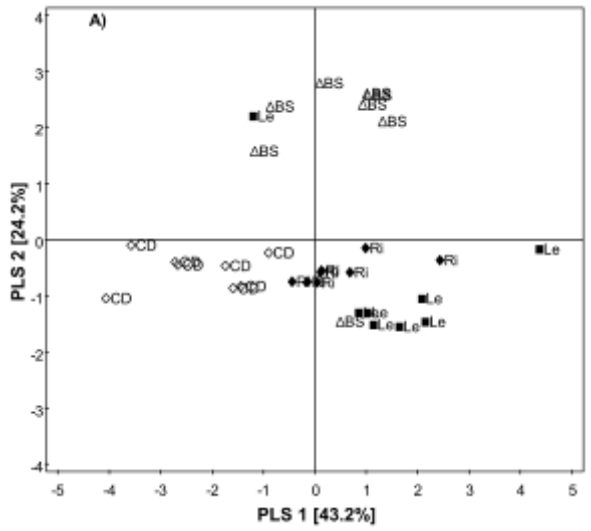
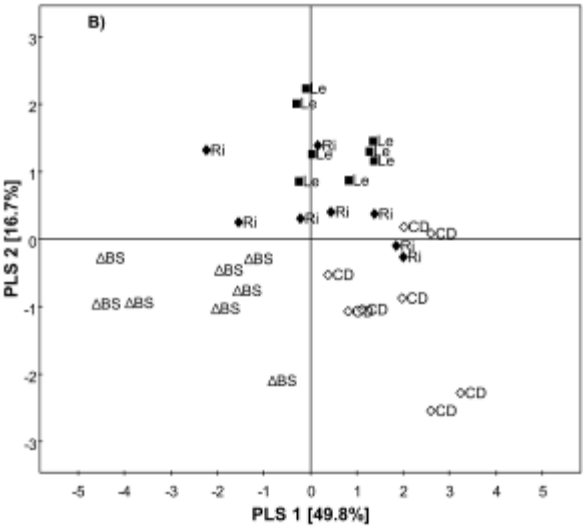
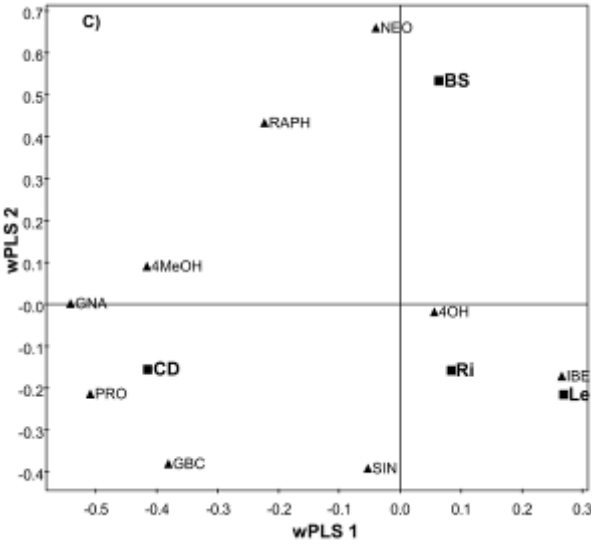


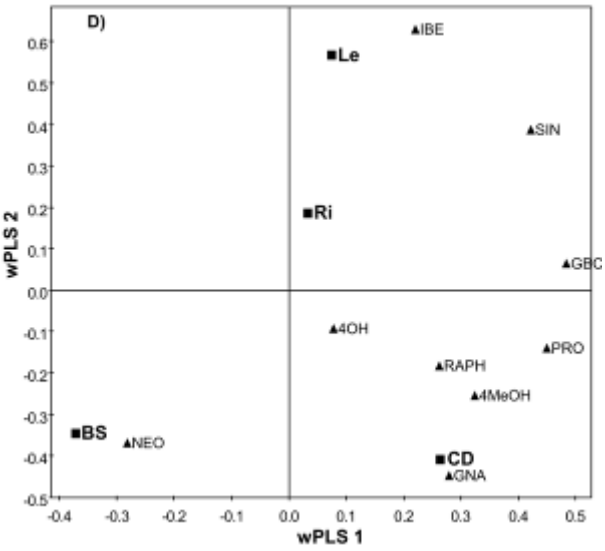
Fig. 4.3 *Abundance of aphids and their parasitoids and predators over time on plants of four Brassica oleracea cultivars in a common garden experiment in 2008. (A) the aphid Brevicoryne brassicae (mean number of aphids per plant ± SE); (B) other aphids (mean number of aphids per plant ± SE); (C) the aphid parasitoid Diaeretiella rapae (mean number of parasitized B. brassicae per plant ± SE); (D) the syrphid predator Episyrphus balteatus (mean number of larvae per plant ± SE); (E) the lacewing predator Chrysoperla carnea (mean number of eggs per plant ± SE).*

In both years, insect abundance was correlated with GLS profile (2008: 1 PLS-component, R_2X = 0.387, R_2Y = 0.134, Q_{2cum} = 0.045; 2009: 1 PLS-component, R_2X = 0.294, R_2Y = 0.199, Q_2 = 0.101; Figs 4.5A and 4.5B). Based on the highest VIP-values in both years, glucoiberin influenced abundance of herbivores and their natural enemies most. In general, plants with low concentrations of glucoiberin and high concentrations of most other


A)
PLS 2 [24.2%]
PLS 1 [43.2%]


B)
PLS 2 [16.7%]
PLS 1 [49.8%]


C)
wPLS 2
wPLS 1
BS
CD
Ri
Le
NEO
RAPH
4MeOH
GNA
4OH
PRO
GBC
SIN
IBE


D)
wPLS 2
wPLS 1
Le
Ri
BS
CD
IBE
SIN
GBC
4OH
PRO
RAPH
4MeOH
NEO
GNA

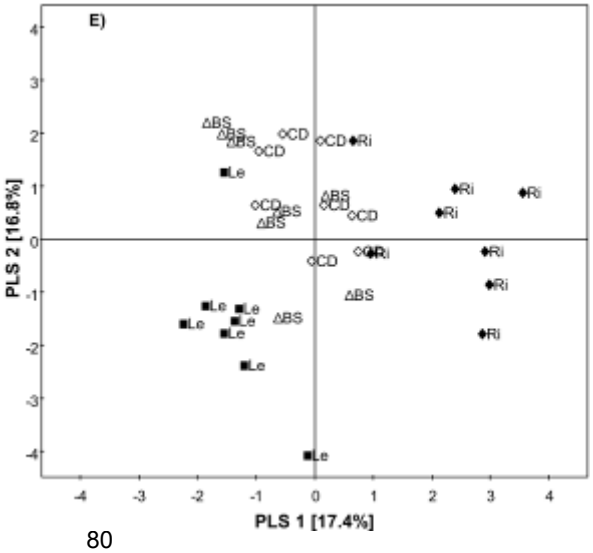

E)
PLS 2 [16.8%]
PLS 1 [17.4%]

Fig. 4.4 *Multivariate analysis of the glucosinolate (GLS) and amino acid profiles of field plants of four Brassica oleracea cultivars: Rivera (Ri), Lennox (Le), Christmas Drumhead (CD) and Badger Shipper (BS). Along the axes, between brackets, the percentage of variation explained is indicated. Score plot of the first two components of projection to latent structures-discriminant analysis (PLS-DA) based on the GLS profiles in 2008 (A) and 2009 (B); Loading plot of the first two components of PLS-DA based on the relative concentrations of different GLS compounds in 2008 (C) and 2009 (D); Score plot of the first two components of PLS-DA based on the amino acid profiles in 2008 (E). Note that in (E) only the first principal component is significant according to the model. Score plots show the distinction in chemical profiles among the cultivars. Loading plots show the contribution of each of the chemical compounds to the discrimination between the cultivars. Aliphatic GLS: GNA = gluconapin, IBE = glucoiberin, IBV = glucoiberverin, PRO = progoitrin, RAPH = glucoraphanin, SIN = sinigrin. Indole GLS: GBC = glucobrassicin, NEO = neo-glucobrassicin, 4MeOH = 4-methoxyglucobrassicin, 4OH = 4-hydroxyglucobrassicin.*

GLS, like gluconapin, progoitrin, and glucoraphanin, harboured higher herbivore and natural enemy abundances (Figs 4.5A and 4.5B). The abundance of the natural enemies was highly correlated with the abundance of their host or prey (Fig. 4.5A and 4.5B).

4.3.2.2 Amino acids and sugars

The total concentrations of amino acids in 2008 did not differ among plant cultivars (Table 4.1 and S4.5). Amino acid profiles on the other hand, differed among cultivars (cultivar effect, 1 PLS-DA principal component, R_2 = 0.174, R_2Y = 0.259, Q_2 = 0.124), although the variation that was explained by the model was small (17.4%; Fig. 4.4E). PLS analysis did not show a correlation of insect abundance with amino acid profiles.

Three sugars were identified: glucose, fructose and sucrose. There were no differences in total sugar concentrations (Table 4.1 and S4.5), or in the sugar profiles among the cultivars. Furthermore, the PLS analysis did not show a correlation between insect abundance and sugar profiles.

4.3.2.3 Plant morphology

Plant developmental stage index values differed among the four cultivars (repeated mixed models: cultivar: *d.f.* = 3, F = 181.06, P < 0.001; time: *d.f.* = 13, F = 390.63, P < 0.001; interaction *d.f.* = 39, F = 11.35, P < 0.001). Christmas Drumhead and Badger Shipper developed faster than Lennox, and

Table 4.1 Mean (± SE) of plant characteristics of each of four *Brassica oleracea* cultivars in the field

Variable	Leaf age	*Brassica oleracea* cultivar			
2008[a]		**Rivera**	**Lennox**	**Christmas Drum-head**	**Badger Shipper**
Indole GLS (µmol g^{-1})[b]	Intermediate	10.25 ± 1.35 **ab**	7.12 ± 1.21 **a**	13.71 ± 0.96 **b**	9.69 ± 1.23 **ab**
Aliphatic GLS (µmol g^{-1})[b]	Intermediate	25.67 ± 2.80 **a**	23.74 ± 2.95 **a**	20.53 ± 2.64 **a**	18.87 ± 2.15 **a**
Total GLS (µmol g^{-1})	Intermediate	35.93 ± 4.09 **a**	30.87 ± 3.73 **a**	34.32 ± 3.14 **a**	28.57 ± 2.80 **a**
Total amino acids (mmol g^{-1})	Intermediate	1.22 ± 0.08 **a**	1.28 ± 0.07 **a**	1.13 ± 0.05 **a**	1.21 ± 0.07 **a**
Total sugars (µmol g^{-1})	Intermediate	214.10 ± 18.25 **a**	257.44 ± 17.64 **a**	214.80 ± 24.34 **a**	232.90 ± 18.17 **a**
2009[c]					
Indole GLS (µmol g^{-1})[b]	Young	7.20 ± 1.56 **de**	10.15 ± 2.36 **e**	9.14 ± 2.16 **e**	8.93 ± 1.10 **e**
	Intermediate	4.77 ± 1.22 **bcde**	4.39 ± 0.46 **cde**	8.28 ± 0.81 **e**	2.35 ± 0.68 **abc**
	Old	1.88 ± 0.25 **abc**	1.51 ± 0.34 **ab**	2.45 ± 0.46 **abcd**	0.85 ± 0.15 **a**
Aliphatic GLS[c] (µmol g^{-1})[b]	Young	9.41 ± 1.48 **de**	7.58 ± 0.49 **de**	7.63 ± 0.99 **de**	9.72 ± 0.98 **e**
	Intermediate	7.87 ± 1.47 **de**	12.26 ± 1.18 **e**	6.93 ± 0.80 **de**	3.54 ± 0.93 **cd**
	Old	1.25 ± 0.48 **ab**	1.85 ± 0.25 **bc**	1.17 ± 0.87 **a**	0.42 ± 0.14 **a**
Total GLS (µmol g^{-1})	Young	16.62 ± 2.69 **d**	17.73 ± 2.14 **d**	16.77 ± 2.50 **d**	18.65 ± 1.05 **d**
	Intermediate	12.64 ± 2.54 **cd**	16.65 ± 0.94 **d**	15.21 ± 1.31 **d**	5.89 ± 1.51 **bc**
	Old	3.13 ± 0.64 **ab**	3.35 ± 0.48 **b**	3.62 ± 1.02 **ab**	1.26 ± 0.19 **a**

Fresh weight shoot (kg)	-	2.15 ± 0.11 **ab**	2.36 ± 0.14 **a**	1.87 ± 0.09 **b**	2.39 ± 0.13 **a**
Epicuticular wax (µg cm^{-2})[d]	Intermediate	55.57 ± 7.70 **b**	41.82 ± 7.86 **ab**	37.36 ± 2.38 **a**	41.47 ± 2.96 **ab**
Leaf toughness (force to break leaf; kg)	Young	0.547 ± 0.031 **ef**	0.786 ± 0.042 **g**	0.309 ± 0.018 **a**	0.422 ± 0.010 **bcd**
	Intermediate	0.649 ± 0.035 **fg**	0.694 ± 0.030 **g**	0.357 ± 0.010 **ab**	0.420 ± 0.016 **bc**
	Old	0.523 ± 0.027 **def**	0.474 ± 0.020 **cde**	0.320 ± 0.012 **a**	0.324 ± 0.014 **a**
Reflectance (%) at 330 nm	Young	12.85 ± 0.53 **cde**	10.64 ± 0.73 **abcd**	11.34 ± 1.03 **abcd**	16.01 ± 1.48 **e**
	Intermediate	13.27 ± 0.87 **de**	12.24 ± 0.82 **cde**	11.46 ± 0.56 **bcd**	11.84 ± 0.95 **bcde**
	Old	9.66 ± 0.92 **abcd**	9.14 ± 0.99 **abc**	8.13 ± 0.60 **ab**	7.70 ± 0.38 **a**
Reflectance (%) at 550 nm	Young	21.65 ± 0.65 **def**	24.32 ± 0.52 **f**	18.33 ± 1.13 **cd**	22.42 ± 1.13 **ef**
	Intermediate	20.25 ± 0.63 **cde**	22.83 ± 0.80 **ef**	14.94 ± 0.61 **ab**	19.58 ± 0.56 **cde**
	Old	18.60 ± 0.48 **cd**	20.74 ± 0.80 **cdef**	12.44 ± 0.62 **a**	17.84 ± 0.53 **bc**
Reflectance (%) at 680 nm	Young	10.38 ± 0.30 **bc**	10.07 ± 0.24 **b**	7.94 ± 0.33 **a**	11.98 ± 0.68 **c**
	Intermediate	10.89 ± 0.55 **bc**	11.26 ± 0.38 **bc**	7.71 ± 0.28 **a**	11.17 ± 0.24 **bc**
	Old	10.26 ± 0.26 **bc**	11.37 ± 0.41 **bc**	6.50 ± 0.27 **a**	10.53 ± 0.20 **bc**

[a] Different letters within a variable (one row) denote differences in means between the four cultivars as analysed by post-hoc Tukey tests. [b] Glucosinolates (GLS) were grouped according to their biosynthetic origin. [c] Different letters within a variable (three rows) denote differences in means between the nine cultivar x leaf age combinations as analysed by post-hoc Tukey tests. [d] Pair-wise differences in amount of epicuticular wax between cultivars were analysed by Mann-Whitney U-tests as assumptions of normality were violated.

all three cultivars developed faster than Rivera.

Furthermore, the four plant cultivars differed in all measured morphological traits (Table 4.1 and S4.5). Leaves of Rivera carried larger amounts of leaf surface wax than leaves of Christmas Drumhead. Leaves of Rivera and Lennox were tougher than the leaves of Christmas Drumhead and Badger Shipper. The fresh weight of Christmas Drumhead plants was lower than that of Lennox and Badger Shipper. In general, leaves of Christmas Drumhead had a lower reflectance at the three different wavelengths tested than leaves of the other cultivars. Similar for all cultivars, young leaves were tougher and had a higher reflectance at the different wavelengths than old leaves (Table 4.1 and S4.5).

Insect abundance was correlated with morphological traits (1 PLS-component, R_2X = 0.470, R_2Y = 0.270, Q_2 = 0.174; Fig. 4.5C). Plants with tougher leaves, larger amounts of leaf surface wax, a higher biomass, a higher reflectance at the different wavelengths and a lower value of the developmental stage index harboured smaller populations of all insect species (Fig. 4.5C). Based on the highest VIP-value, leaf toughness influenced insect abundance most.

4.3.3 Parasitisation and predator oviposition responses to inoculated herbivores

Parasitism rates of experimentally released *P. xylostella* caterpillars and *B. brassicae* aphids changed over the season (time effect, GLM, $P < 0.001$ for both species in both years). Within the natural fluctuations of parasitism pressure, cultivars differed in the fraction of *P. xylostella* caterpillars that were parasitized by *D. semiclausum* in 2008 (cultivar effect, GLM: *d.f.* = 3, deviance ratio = 4.18, P = 0.007): a larger fraction was parasitized on Christmas Drumhead and Badger Shipper, than on Rivera (Fig. 4.6A). In 2009, there were no differences among the cultivars (cultivar effect, GLM: *d.f.* = 3, deviance ratio = 2.24, P = 0.085; Fig. 4.6A). The average fraction of *D. semiclausum* females emerging from the parasitoid pupae was 0.60 ± 0.02 in 2008 and 0.51 ± 0.02 in 2009 and was similar for the four cultivars (cultivar effect, GLM: 2008: *d.f.* = 3, deviance ratio = 0.20, P = 0.893; 2009: *d.f.* = 3, deviance ratio = 2.30, P = 0.079).

In 2008, we only compared parasitisation of aphids on field plants of Christmas Drumhead and Badger Shipper, because almost no aphids on Rivera and Lennox survived (data not shown). There was no difference in the

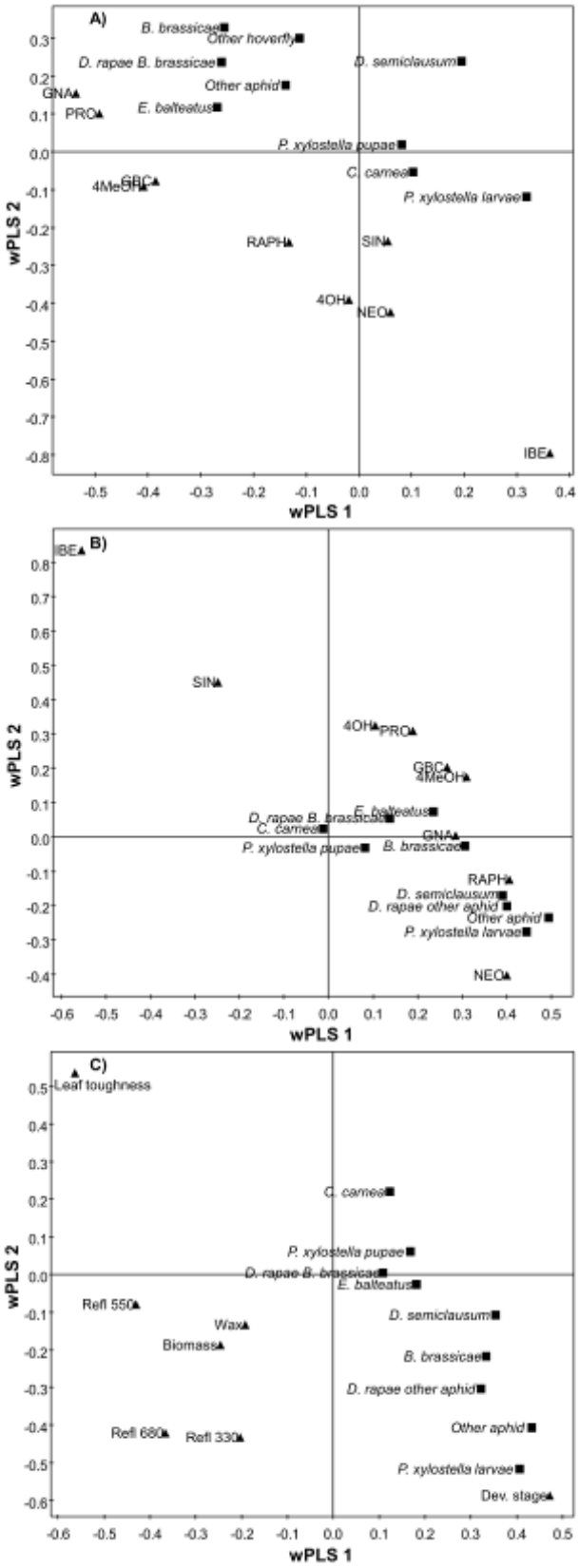


Fig. 4.5 *Multivariate correlations between insect abundance and glucosinolate (GLS) profiles and morphological traits of Brassica oleracea in 2008 and 2009. Loading plot of the first two components of PLS based on the concentrations of different GLS compounds in 2008 (A) and 2009 (B); loading plot of the first two components of PLS based on the different morphological traits in 2009 (C). Plots show the contribution of each GLS compound or morphological trait to abundance of the insect species. Note that for all plots only the first principal component is significant according to the model. Selected insect species are: caterpillars and pupae of the lepidopteran Plutella xylostella, cocoons of the Plutella-attacking parasitoid Diadegma semiclausum, the aphid Brevicoryne brassicae, other aphids, mummies of the aphid parasitoid Diaeretiella rapae with B. brassicae as host (labelled as D. rapae B. brassicae) or other aphids as host (labelled as D. rapae other aphid), larvae of the syrphid predator Episyrphus balteatus, larvae of other predatory hoverflies and eggs of the lacewing predator Chrysoperla carnea. Aliphatic GLS: GNA = gluconapin, IBE = glucoiberin, PRO = progoitrin, RAPH = glucoraphanin, SIN = sinigrin. Indole GLS: GBC = glucobrassicin, NEO = neo-glucobrassicin, 4MeOH = 4-methoxyglucobrassicin, 4OH = 4-hydroxyglucobrassicin. Refl = reflectance at three different wavelengths (330, 550 and 680 nm). Dev. stage = developmental stage index*

fraction of aphids parasitized on these cultivars (cultivar effect, GLM: *d.f.* = 1, deviance ratio = 0.88, *P* = 0.352; Fig. 4.6B). In 2009, a smaller fraction of aphids was parasitized on Badger Shipper than on the other cultivars (cultivar effect, GLM: *d.f.* = 3, deviance ratio = 8.80, *P* < 0.001; Fig. 4.6B).

Most *E. balteatus* and *C. carnea* eggs were laid between weeks 29 and 34 (mid-July to mid-August) in 2009. There was no difference in the number of *E. balteatus* eggs or *C. carnea* eggs among the cultivars (cultivar effect, Kruskal-Wallis H-test: *E. balteatus*: *d.f.* = 3, χ^2 = 4.27; *P* = 0.233; *C. carnea*: *d.f.* = 3, χ^2 = 0.49; *P* = 0.922; Fig. 4.6C).

The fraction recovery of experimentally released herbivores from the field changed over time but did not differ among cultivars (Table S4.6).

4.4 Discussion

Our data clearly show that intraspecific variation in chemistry and morphology of *B. oleracea* affects the abundance of insect herbivores and their natural enemies in the field. Although plant-mediated bottom-up and top-down forces interact in complex ways our data suggest that, in our study system, bottom-up forces are more important for herbivore abundance than plant-mediated top-down forces.

4.4.1 Effects of intraspecific plant variation on herbivore and natural enemy abundance

Brassica oleracea cultivars that supported the largest numbers of herbivores (Christmas Drumhead and Badger Shipper) also supported the largest numbers of parasitoids and predators of these herbivores, suggesting a direct translation of effects on herbivores to effects on their natural enemies (Sznajder and Harvey 2003; Bukovinszky et al. 2008). The herbivore species *P. xylostella* and *B. brassicae*, which differ fundamentally in their feeding mode (leaf chewing and phloem sucking, respectively), showed similar responses to intraspecific plant differences. The difference in abundance between the *B. oleracea* cultivars was, however, greater for *B. brassicae* than for *P. xylostella*, which is in agreement with the study by Poelman et al. (2009b). This could be due to the rapid, exponential population growth of *B. brassicae* that allows for a faster increase in abundance on the most suitable host plant (Broekgaarden et al. 2008). Despite differences in feeding strategy between parasitoid wasps and predators, the statistical analyses showed similar abundance patterns of both functional groups of natural enemies.

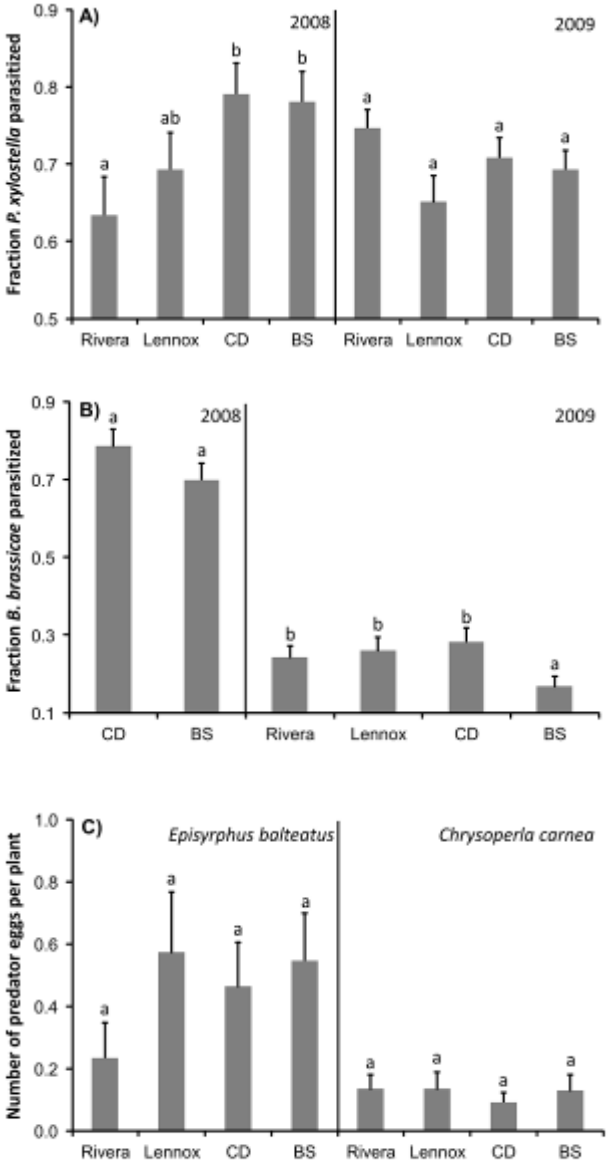


Fig. 4.6 *Mean (+ SE) fraction of parasitized herbivores and number of predator eggs on plants of four Brassica oleracea cultivars that were inoculated with a controlled number of herbivores. Fraction of Plutella xylostella caterpillars (A) and Brevicoryne brassicae aphids (B) that were parasitized in 2008 and 2009; and number of eggs of Episyrphus balteatus and Chrysoperla carnea in 2009 (C). Cultivars: Rivera, Lennox, Christmas Drumhead (CD) and Badger Shipper (BS). Within a study year or predator species, different letters indicate differences among the cultivars.*

4.4.2 Bottom-up effects on herbivore and natural enemy abundance

We recorded a correlation between the GLS profile of a cultivar and the insect abundance it harboured, which was consistent for both years. Insect abundance was low on plants with high concentrations of the aliphatic GLS glucoiberin, *i.e.* the cultivars Rivera and Lennox. The same negative correlation between glucoiberin and herbivore abundance was reported by

Poelman et al. (2009b). The *B. oleracea* cultivars also differed in total GLS concentrations, but these differences were not consistent between the two years and did not explain variation in insect abundance. Based on our results, we suggest that specific GLS can shape insect abundance more strongly than total concentrations of these compounds. Herbivore species may show differential responses to different combinations of specific compounds, and to confer resistance to many different attackers, plants may maintain a large variation in their chemical profile (Jones and Firn 1991; Newton et al. 2009b; Poelman et al. 2009b), without suffering from trade-offs between these different chemical resistance traits (Koricheva et al. 2004).

Concentrations or profiles of foliar amino acids and sugars did not provide an explanation for the differences in insect abundance among the cultivars. Thus, in the tritrophic system studied here, primary metabolites do not appear to determine insect abundance. Although a close relationship between plant nutritional value and insect performance has often been suggested, no general trends for this relationship have been established (Cole 1997; Schoonhoven et al. 2005 and references therein).

There was a strong correlation between insect abundance and morphological traits of the cultivars. Rivera and Lennox, characterized by tougher leaves, larger amounts of leaf surface wax, a higher reflectance at the different wavelengths (representative of greener leaves with more leaf surface wax), and a slower development, harboured smaller insect populations than Christmas Drumhead and Badger Shipper. The strongest negative correlation with insect abundance was observed for leaf toughness. Leaf toughness is thought to be a critical biomechanical property affecting both chewing and sucking herbivores, and a negative association between the performance of several herbivores and leaf toughness has been documented (Read and Stokes 2006; Clissold et al. 2009). In a meta-analysis, Carmona et al. (2011) proposed that variation in herbivore susceptibility is more strongly correlated with genetic variation in plant morphological and life-history traits than with genetic variation in concentrations of secondary metabolites.

4.4.3 Top-down effects on herbivore abundance

Parasitisation and predator oviposition are a result of several processes: attraction of the natural enemy (*e.g.* by herbivore-induced plant volatiles), arrestment of the natural enemy by plant or host/prey odours and contact cues, and actual oviposition in or near the host/prey. In this field study we did

not assess differences in volatile emission among the *B. oleracea* cultivars. We inoculated plants with herbivores and assessed differences in the fraction of herbivores parasitized and in predator oviposition among the *B. oleracea* cultivars. We used a controlled number of herbivores to rule out host-density-dependent effects on natural enemies, thus allowing us to test specifically for differences in attraction and arrestment of natural enemies among cultivars.

The observed differences in parasitisation and oviposition among the cultivars were not consistent between years, were mostly not significant, and did not explain the observed differences in natural herbivore abundance among cultivars as observed in the insect abundance experiment. If natural enemies would mainly affect herbivore abundance (top-down control), one would expect that the least attractive cultivars would harbour the most herbivores, but this was not the case. The opposite trend was observed: herbivore abundance was highest on cultivars that appeared most attractive in the inoculation experiment. Therefore, we infer that top-down forces did not affect herbivore abundance to a large extent.

4.4.4 Bottom-up versus top-down effects on herbivore abundance

The abundance of herbivores on different *B. oleracea* cultivars, as observed in our study, resulted most likely from a combination of bottom-up and plant-mediated top-down forces. Our results indicate, however, that plant traits that acted as bottom-up forces were more important for herbivore abundance than plant-mediated top-down forces. Although there is no natural selection on cultivated plants, there is probably selection by cultivated plants on the associated insect species, which results in effects on the insect community. Our results are in agreement with a similar study that tested the interaction of wild *B. oleracea* populations, instead of cultivated varieties, with the aphid *B. brassicae* and the parasitoid *D. rapae* (Newton et al. 2009a). Furthermore, our results correspond with the general view that in terrestrial systems top-down forces are considered to be weaker than bottom-up forces (Hunter and Price 1992; Schmitz et al. 2000; Halaj and Wise 2001; Walker et al. 2008). This view is based on a number of factors, such as the complexity and high species diversity of terrestrial ecosystems that reduce the effect of a single carnivore species, and the potential for compensation of a decrease in the herbivore population by an increase in plant consumption by the remaining herbivores (Schmitz et al. 2000).

4.4.5 Conclusion

Our results show not only that concentrations of secondary metabolites and morphological traits of *B. oleracea* plants affect the abundance of herbivores consistently over different seasons, but that these traits also affect the abundance of natural enemies of these herbivores, thus leading to bottom-up effects of plant traits on the third trophic level. Although the abundance of herbivores is most likely influenced by a combination of bottom-up and top-down forces, it appears that bottom-up forces were relatively more important than plant-mediated top-down forces.

We call for further unravelling of the complex interactions between bottom-up and top-down forces that structure plant-based arthropod communities. Therefore, long-term studies that address the relative contribution of bottom-up and top-down effects on insect communities simultaneously and that include analyses of secondary metabolites and morphological plant traits, are required to improve the evaluation of the relative importance of these effects on insect communities.

4.5 Acknowledgements

We thank two anonymous reviewers for constructive comments on an earlier version of the manuscript; Rieta Gols and Roland Mumm for help with the statistical analyses; CGN and Bejo Zaden for providing seeds of the *B. oleracea* cultivars, Leo Koopman, Léon Westerd and André Gidding for rearing the insects and Unifarm, especially John van der Lippe, Rinie Verwoert, Andre Maassen and Alex Super, for plant rearing and maintenance of the field site. This work was supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (number 838.06.010).

5

Effects of soil organisms on aboveground multitrophic interactions are consistent between plant genotypes mediating the interaction

Patrick Kabouw*, **Martine Kos***, Sandra Kleine, Elke A. Vockenhuber, Joop J.A. van Loon, Wim H. van der Putten, Nicole M. van Dam and Arjen Biere

** Both authors contributed equally to the manuscript*

Published in a slightly different form in *Entomologia Experimentalis et Applicata* 139: 197-206 (2011)

Abstract

Belowground communities can affect interactions between plants and aboveground insect communities. Such belowground-aboveground interactions are known to depend on the composition of belowground communities, as well as on the plant species that mediates these interactions. However, it is largely unknown whether the effect of belowground communities on aboveground plant-insect interactions also depends on genotypic variation within the plant species that mediates the interaction. To assess whether the outcome of belowground-aboveground interactions can be affected by plant genotype we selected two white cabbage cultivars [*Brassica oleracea* L. convar. *capitata* var. *alba* (Brassicaceae)]. From previous studies, it is known that these cultivars differ in their chemistry and belowground and aboveground multitrophic interactions. Belowground, we inoculated soils of the cultivars with either nematodes or microorganisms and included a sterilized soil as a control treatment. Aboveground, we quantified aphid [*Brevicoryne brassicae* L. (Hemiptera: Aphididae)] population development and parasitoid [*Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae)] fitness parameters. The cultivar that sustained highest aphid numbers also had the best parasitoid performance. Soil treatment affected aphid population sizes: microorganisms increased aphid population growth. Soil treatments did not affect parasitoid performance. Cultivars differed in their amino acid concentration, leaf relative growth rate, and root, shoot, and phloem glucosinolate (GLS) composition but showed similar responses of these traits to soil treatments. Consistent with this observation, no interactions were found between cultivar and soil treatment for aphid population growth or parasitoid performance. Overall, the aboveground community was more affected by cultivar, which was associated with GLS profiles, than by soil community.

5.1 Introduction

Recently, it has become widely acknowledged that belowground communities can have profound effects on aboveground insect communities through plant-mediated interactions (Wardle et al. 2004a; Bardgett and Wardle 2010). Aboveground herbivore performance can be either stimulated or reduced by root-associated communities. Several studies recorded that root-associated communities increased (Gange and Brown 1989; Poveda et al. 2003; Bezemer et al. 2005) or decreased aboveground herbivore fitness parameters (Sell and Kuosell 1990). This paradox may be understood if we consider the various groups of belowground organisms that were used in these studies and the nature of their interactions with plants.

Soil organisms can be either beneficial or detrimental to the plant and the associated aboveground herbivores. By mobilizing nutrients, soil organisms can increase plant quality and the fitness of aboveground herbivores (Eisenhauer et al. 2010; Orwin et al. 2010). However, soil organisms that inflict damage to the plant can also up-regulate defensive compounds in the plant and thereby influence aboveground herbivores in a negative way (Wurst et al. 2006). Interactions that underlie linkages between belowground and aboveground communities via the plant may also be less direct (Megias and Müller 2010). For instance, carnivorous soil organisms potentially suppress the abundance of soil organisms mobilizing nutrients, thus influencing plant quality and aboveground communities. Consequently, the strength and direction of belowground-aboveground interactions are context-specific; therefore, the outcome of the interactions is likely to be less predictable when recorded at the community level than at the individual level (Wardle 2002).

An additional layer of complexity affecting the outcome of belowground -aboveground interactions comes from variation at the plant level. Responses of plants, when exposed to belowground communities, often differ between species (van Dam and Raaijmakers 2006). Such differences may result in differential effects on higher trophic levels aboveground. For instance, the effect of soil community on aphid performance differed among grass species and these differential effects cascaded up to *Aphidius colemani* Viereck parasitizing the aphid *Rhopalosiphum padi* L. (Bezemer et al. 2005). Hence, both the soil community and the plant species mediating the interaction can significantly influence the response of aboveground insect communities.

Plant genotypic variation is well known to affect plant-insect

interactions (Crutsinger et al. 2006) and recent studies suggest that the outcome of belowground-aboveground interactions may also depend on which genotype mediates the interactions. For example, in *Plantago lanceolata* L. chemotypes with different iridoid glycoside concentrations, exposure to wireworms changed the defensive compounds in a chemotype-specific way (Wurst et al. 2008). On the other hand, chemical profiles of two *Barbarea vulgaris* R. Br. chemotypes responded similarly to root feeding by *Delia radicum* L.(van Leur et al. 2008). However, both studies used relatively simple belowground communities, with only one root herbivore instead of a more complex community. Additionally, both studies did not examine effects on higher trophic levels aboveground, so that biological implications of intraspecific variation on higher trophic levels in belowground-aboveground interactions remain unknown.

Wild and cultivated *Brassica* species (Brassicaceae) are widely used as model species for belowground-aboveground interactions (Poveda et al. 2003; van Dam and Raaijmakers 2006; Soler et al. 2010). Brassicaceae are characterized by a structurally diverse group of defensive compounds called glucosinolates (GLS), which upon herbivory hydrolyse to (iso)thiocyanates and nitriles, that are toxic to a variety of insects (Mithen 2001). GLS in aboveground tissues of *Brassica* species are known to respond to belowground herbivory. For example, *D. radicum* increases GLS concentrations in shoots of *Brassica nigra* L. (van Dam and Raaijmakers 2006), whereas nematodes reduce GLS concentrations in *Brassica oleracea* L. (Wurst et al. 2006). Differences between genotypes in their response of these defensive compounds to belowground organisms could thus potentially result in differences in the outcome of belowground-aboveground interactions.

The objective of this study was therefore to examine whether plant intraspecific variation can affect the outcome of belowground-aboveground plant-mediated interactions up to the third trophic level. Making use of previous studies (Poelman et al. 2009b; Kabouw et al. 2010a), we chose to examine belowground-aboveground interactions in two white cabbage cultivars that were known to differ amongst others in their root and shoot GLS profiles and in their interactions with nematodes belowground and herbivores aboveground. We assessed whether soil treatments (nematodes or microorganisms) affect aboveground aphid populations of the specialist aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae). The performance of this aphid is known to be negatively affected by specific GLS in plants (Cole 1997; Mewis et al. 2005;

Kim et al. 2008; Kissen et al. 2009). Therefore, we examined soil-treatment mediated changes in total leaf and phloem GLS concentrations as well as leaf and phloem GLS profiles. *Brevicoryne brassicae* is known to sequester GLS for its own defence against predators (Francis et al. 2001b; Jones et al. 2001; Kazana et al. 2007). We expected that if soil organisms are able to modify the GLS profiles in the *Brassica* cultivars, it could be reflected in changes in GLS profiles of the aphid as well. Such changes in herbivore defensive chemistry have previously been shown to affect higher trophic levels (Müller 2009). We examined whether higher trophic level interactions, between the aphid and its parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae), differed among cultivars or soil treatments. In addition to GLS, we measured primary metabolites, amino acids, as well as plant biomass parameters (relative growth rate, root and shoot biomass), as a measure for plant quality which might increase or decrease for instance due to the ability of soil organisms to mobilize nutrients (Eisenhauer et al. 2010; Orwin et al. 2010).

5.2 Materials and methods

5.2.1 Experimental set-up

Seeds of two white cabbage cultivars – Rivera (breeder and seed source: Bejo Zaden, Warmenhuizen, The Netherlands) and Badger Shipper (breeder: University of Wisconsin, Madison, Wisconsin, USA; seed source: Centre of Genetic Resources, Wageningen, The Netherlands) – were germinated for 10 days in a growth cabinet, at a 16:8 h light:dark (L:D) photoregime (temperature 25:15 °C). A total of 90 seedlings per cultivar were transferred to pots filled with 2 kg of a loamy, sandy mineral soil (0.13% N, 2.1% C, C/N = 16.7; particle size distribution: 3% <2 µm; 17% 2-63 µm; 80% >63 µm), which had been sieved (mesh size 5 mm) and gamma-sterilized at 25 kGrey (Isotron, Ede, The Netherlands). Pots were relocated to a greenhouse with a 16:8 h L:D photoregime (temperature 21:16 °C) hours, regulated by sodium lamps to maintain a minimum photoactive radiation of 225 µmol m^{-2} s^{-1}. Plants were randomly divided over three treatments (30 replicates per cultivar per treatment). The treatments per cultivar consisted of (1) a control group of 30 plants without a soil inoculum, (2) 30 plants supplied with a nematode inoculum, and (3) 30 plants with only a microorganism inoculum.

To obtain a microorganism solution, 11 kg of the same unsterilized soil in which the plants were potted was suspended with 11 L of deionized water and incubated for 24 h at room temperature. The supernatant was passed over

five sieves (1 × mesh size 75 µm, 3 × 45 µm, and 1 × 10 µm), which retained nematodes but allowed passing of most microorganisms (Bezemer et al. 2005). Aliquots of 70 ml were added to each pot of the microorganism treatment. Nematodes were extracted from the same soil as described above (but unsterilized) by filtering it over four sieves (1 × 75 µm, 3 × 45 µm) which retained the nematodes. The residue with the nematodes was purified by incubating for 24 h at room temperature on two milk filters suspended between clamps. This allowed nematodes to migrate into tap water; it was not possible to use deionized water because the osmotic potential could kill the nematodes. The resulting solution, inevitably containing microorganisms, was pooled and nematodes were counted in 10 subsamples to determine the density. To add 2 000 nematodes per kg of soil, which is an average density for the soil type used in this experiment (Bezemer et al. 2004), 40 ml of the solution was required and added to the pots assigned for nematode treatment. We added 30 ml of tap water to supply the same amount of liquid as in the control and microorganism treatments. Control plants were supplied with 70 ml of demineralized water.

5.2.2 Aphid population growth and parasitoid performance

Brevicoryne brassicae were obtained from cultures maintained in a greenhouse on Brussels sprouts (*B. oleracea*). Parental aphids from the stock rearing were exposed to additional plants of the respective treatment and allowed to larviposit for 24 h on these plants, after which they were removed. When the new-born nymphs reached the second instar, 10 of them were put on two young leaves of 10 plants per treatment and cultivar. Plants were placed individually in gauze nets. Aphid population sizes on these plants were monitored twice a week for 29 days, starting 8 days after introduction of the aphids. After 29 days of infestation, all aphids were collected from the plant for GLS analysis and stored at –20 °C until freeze-drying. The extraction of the GLS is described below.

To monitor the effect on the third trophic level, 10 plants per treatment and cultivar were infested with 10 second instars that had been parasitized by the solitary endoparasitoid *D. rapae*. These aphids were reared on plants of the respective treatment, parasitized, and then again transferred to plants of the respective treatment. An aphid was considered parasitized when the female parasitoid inserted her ovipositor in the aphid. From the day the first mummy (aphid remains containing a parasitoid pupa) appeared, plants were

surveyed daily for new mummies. Mummies were then transferred into glass vials and from 7 a.m. until 11 p.m. checked every 2 h to record survival rate, egg-to-adult development time, adult dry mass, and sex ratio of the adult parasitoids. Since their collection from a Brussels sprouts field near Wageningen (The Netherlands) the parasitoids were reared in a climate room on *B. brassicae*.

5.2.3 Collecting plant and soil samples

From the start of the experiment until aphid infestation, the first and second leaves were photographed weekly and the size of the leaves was calculated by using a standard reference area, which was included in the photograph. From these photographs the leaf area was determined using WinFolia (Regent Instrument, Ottawa, Ontario, Canada). Based on these measurements, the relative growth rate was estimated as $(\ln \text{area2} - \ln \text{area1})/(t_2 - t_1)$.

Synchronised with the aphid infestation, so 4 weeks after the initiation of soil treatments, 10 plants per treatment and cultivar were harvested. The first, second, and fourth youngest leaf of each plant were pooled and stored at –20 °C for GLS analysis. Additionally, we collected phloem of the third leaf. For this, we followed the procedure outlined in Bezemer et al. (2005) with the modification that we used 2 ml 8 mM EDTA solution, left the leaf 2 h instead of 1 h in this solution, and initially put the leaf for 5 min in an extra EDTA solution to remove any plant chemicals from the wound resulting from detaching the leaf from the plant. The remaining aboveground plant material, including the third leaf, was dried at 70 °C before measuring its dry mass. All plant material for GLS extraction was freeze-dried, weighed, and its dry mass was included in total shoot biomass.

Half of the roots of each plant was cleared from the soil and washed to remove soil particles, dried with filter paper, and stored at –20 °C for GLS analysis. The other half of the roots was removed from the soil, shaken, and stored for 3 days at 4 °C in a dark climate chamber before nematode extraction. Afterwards the roots were dried at 70 °C and they were weighed. The soil not directly adhering to these roots was bagged and stored in the dark for 3 days at 4 °C before nematode extraction. A subsample of the soil adhering to the roots (rhizosphere soil) was frozen at –80 °C for polymerase chain reaction (PCR) analysis and denaturing gradient gel electrophoresis (DGGE).

After 29 days of aphid infestation, the remaining plants were harvested

and the third leaf was, similar to the control plants, used for phloem collection as described above. Plant roots and shoots were separated, dried, and weighed.

5.2.4 Soil community analysis

To classify differences in microbial biodiversity, DNA was isolated from the rhizosphere soil using a PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA) following the producer's instructions. Per treatment and cultivar, we analysed four replicates. PCR-DGGE was performed as described in Kabouw et al. (2010b). We analysed all treatments within a cultivar on the same gel, because it is generally difficult to compare gels.

The nematodes from the roots were extracted with a mistifier according to van Bezooijen (2006). Numbers of root-inhabiting nematodes are expressed per g root dry mass. Free dwelling nematodes were extracted from the soil as described in the experimental set-up. The nematodes that passed through the milk filters were conserved with 4% formaldehyde. We counted all nematodes present in the samples, both free living and root inhabiting. A subset of 150 nematodes was identified to family and genus level with the help of an inverted microscope. Free-living nematode numbers are expressed per 100 g dry mass of soil.

5.2.5 Extraction and analysis of amino acids and GLS

The freeze-dried samples of roots, shoots, and aphids were ground with a Ball mill. Approximately 100 mg of the finely ground material was used for GLS extraction. For further purification of GLS and amino acids, see the study by van Dam and Raaijmakers (2006). For the extraction of GLS and amino acids from the phloem we used a modified protocol. From the initial 2 ml of EDTA solution, 1.5 ml was used for GLS extraction. The solution was boiled in a water bath and subjected to an ultrasonic bath for 15 min to inactivate myrosinase activity and brought directly on the Sephadex column. After elution, the freeze-dried elute was resuspended in 100 µl MilliQ to concentrate the samples. Dilution factors for high-performance liquid chromatography (HPLC) were adjusted accordingly. Amino acids were analysed as in van Dam and Raaijmakers (2006), by taking 20 µl of the EDTA phloem solution. HPLC analyses were performed as described in Kabouw et al. (2010b).

5.2.6 Statistical analysis

Nematode numbers were analysed by two-way ANOVA. DGGE gel banding patterns were analysed by distance based redundancy analysis (db-RDA) as described by Kabouw et al. (2010b). However, data were visualized by correspondence analysis.

Aphid numbers were log transformed and analysed by repeated measures two-way ANOVA. Development time and adult dry mass of the parasitoids were averaged per plant. Generalized linear models (GLM) were used to analyse parasitoid survival and sex ratio. Both survival and sex ratio were analysed as a binomially distributed dependent variable per plant (for survival: Y adult parasitoids surviving out of N recollected aphids on that plant; for sex ratio: Y females out of N adult parasitoids). Two-way ANOVAs were used to assess differences in egg-to-adult development time and adult dry mass of parasitoids. Prior to these analyses, Student's t-tests were used to analyse differences between females and males in egg-to-adult development time and adult dry mass of the parasitoids. As male and female wasps did not differ in development time (t = 0.58, P > 0.5) or adult weight (t = 0.28, P > 0.5) and no interaction was recorded between sex and cultivar, nor between sex and soil treatment, data for male and female wasps were pooled in the analyses.

Total concentrations of GLS and amino acids in plants and aphids were analysed by two-way ANOVA. Aphid and plant GLS profiles and plant phloem amino acid profiles were analysed by Monte Carlo Permutation (MCP) tests in a redundancy analysis (RDA) with cultivar or treatment as environmental variable and GLS as species data. Multivariate interactions of GLS profiles were analysed by MCP tests, *i.e.* to see whether soil treatments are differently ranked within the cultivars, with cultivar and treatment as environmental factors. GLM analyses were performed in GenStat (11th edition; VSN International, Hemel Hempstead, UK), RDA models and MCP tests were performed using CANOCO (version 5; Biometris, Wageningen, The Netherlands), whereas all other statistical tests were performed using STATISTICA (version 8; StatSoft, Tulsa, Oklahoma, USA).

5.3 Results

5.3.1 Aphid population growth

Aphid population development differed both between the two cultivars and between the soil treatments. Aphid populations on plants from microorganism-

inoculated soils increased significantly faster than those on plants from nematode-inoculated or control soils (repeated measures ANOVA: $F_{1,53} = 8.62$, $P < 0.05$). By contrast, aphid populations tended to develop slower on plants from nematode-treated soils than on plants from control soils, but this difference was not statistically significant on either of the cultivars (Fig. 5.1). Overall, the cultivar effect was stronger, with two-fold differences in aphid numbers, than the soil treatment effect (Fig. 5.1). On Rivera, the aphid population growth was considerably slower than on Badger Shipper (repeated measures ANOVA: $F_{2,57} = 21.70$, $P < 0.001$). Aphid population growth rates responded similarly to soil treatments on both cultivars (repeated measures ANOVA, soil treatment*cultivar: $F_{2,57} = 0.87$, $P > 0.05$).

5.3.2 Parasitoid performance

Soil treatment did not affect parasitoid developmental time (two-way ANOVA: $F_{2,54} = 0.29$, $P > 0.5$), adult dry mass (two-way ANOVA: $F_{2,54} = 0.35$, $P = 0.5$), sex ratio (GLM: deviance = 0.68, $P > 0.5$), or survival (GLM: deviance = 0.595, $P > 0.5$).

By contrast, parasitoid development was significantly affected by cultivar. Similar to the aphids, parasitoids developed faster on Badger Shipper (13.11 ± 0.24 days) than on Rivera (13.61 ± 0.29 days; two-way ANOVA: $F_{1,54} = 4.80$, $P < 0.05$). Also adult mass was higher on Badger Shipper (0.090 ± 0.003 mg) than on Rivera (0.080 ± 0.002 mg) (two-way ANOVA: $F_{1,54} = 10.28$, $P > 0.01$). The survival of the parasitoids was similar on both cultivars (GLM: deviance = 2.163, $P > 0.2$; Badger Shipper: 92 ± 4%, Rivera: 97 ± 3%). However, wasps emerging from aphids reared on Rivera had a higher fraction of females than those on Badger Shipper (GLM: deviance = 5.205, $P < 0.05$; 43 ± 7% vs. 25 ± 8% respectively). No significant interaction between cultivar and soil treatment was observed for parasitoid developmental time, adult mass, percentage survival, or for the fraction of females that emerged.

5.3.3 DGGE fingerprints and nematode numbers

For both Rivera and Badger Shipper, DGGE patterns of bacteria and fungi differed significantly between the treated soils on both the first and second axes of the db-RDA (MCP tests, Supporting Information Fig. S5.1). Before aphid exposure, total nematode abundances were significantly higher in nematode-treated soils, both in the bulk and in the rhizosphere soil, as was intended (Table 5.1). The number of phytophagous nematodes also differed

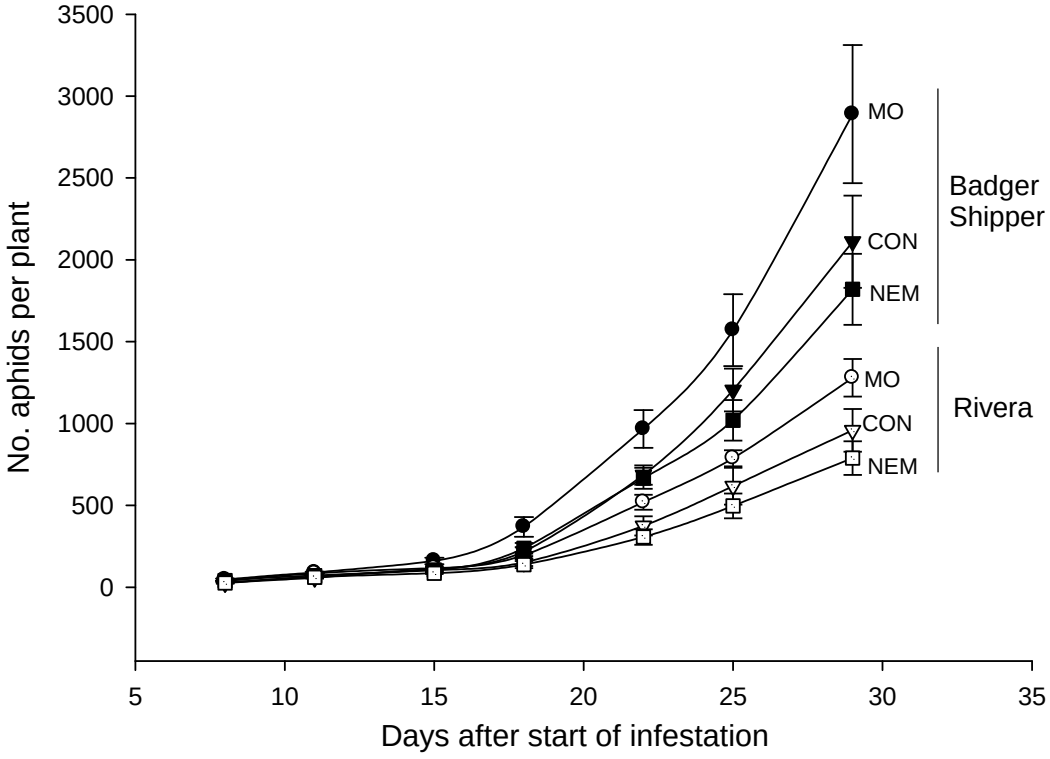


Fig. 5.1 *Aphid population development (mean ± SE number of aphids per plant). Aphids were reared on soils treated with micro-organisms (circles), untreated control soils (inverted triangles), or nematode-treated soils (squares). White symbols represent aphids reared on Rivera and black symbols represent aphids reared on Badger Shipper.*

between treatments and was several magnitudes higher in nematode-treated soils than in microorganism-treated and control soils (Table 5.1). The dominant nematodes belonged to the bacterivorous families Cephalobidae and Rhabditidae. Cultivar affected neither total nematode abundance, nor number of plant parasitic nematodes (Table 5.1). There were also no interaction effects between the cultivars and soil treatments on nematode abundances (Table 5.1).

Table 5.1 Average number (± SD) of nematodes per treatment expressed per 100 g of dry soil or g dry root

Treatment	Total no. of nematodes in the bulk soil	No. plant-parasitic nematodes in the bulk soil	Total no. nematodes per g dry root	No. plant-parasitic nematodes per g dry root
Rivera microorganisms	56 ± 40a	0.6 ± 0.5 a	21.0 ± 15.1 a	0.1 ± 0.1 a
Rivera control	4 ± 1b	0.1 ± 0.1a	1.6 ± 0.5 b	1.0 ± 0.8 a
Rivera nematodes	1189 ± 182 c	11.2 ± 3.7 b	585.2 ± 152.3 c	25.8 ± 20.1 b
Badger Shipper microorganisms	68 ± 61 a	0.2 ± 0.2 a	11.5 ± 10.1 a	0.2 ± 0.2 a
Badger Shipper control	33 ± 23 a	0.0 ± 0.0 a	26.5 ± 25.8 a	0.2 ± 0.2 a
Badger Shipper nematodes	848 ± 101 b	5.7 ± 2.4 b	728.9 ± 248.2 b	42.5 ± 20.1 b
F cultivar	1.8	1.8	0.3	0.5
F treatment	73.8*	13.6*	19.2*	8.8*
F interaction	2.9	1.3	0.2	0.6

Different letters within a column within a cultivar denote significant differences (Tukey post-hoc analysis; P<0.05).

** Significant effect (two-way ANOVA; P<0.05).*

5.3.4 Plant chemistry

5.3.4.1 GLS

GLS concentrations in the phloem, leaves, and roots were not significantly affected by soil treatment (Table S5.1). Also the GLS profiles were not significantly affected by soil treatment, in leaves (MCP: $P > 0.5$; Fig. 5.2A), roots (MCP: $P > 0.1$), and in the phloem, before (MCP: $P > 0.1$) or after aphid infestation (MCP: $P > 0.1$).

Analogous to the aphid population growth data, differences in GLS concentrations and profiles were much more pronounced between cultivars than between soil treatments. Phloem GLS concentrations were significantly higher in Badger Shipper than in Rivera at the first sampling, whereas this was reverse at the second sampling (Table S5.1). Also the GLS profiles in the phloem differed between the cultivars, both before and after aphid infestation (MCP: both $P < 0.01$). Indole GLS, mainly 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin, dominated in the phloem of Badger Shipper, which had the highest aphid numbers, whereas in Rivera aliphatic GLS, mainly gluconapin and sinigrin, dominated. No interaction between soil treatment and cultivar was recorded for GLS profiles at either the initial (MCP: $P > 0.9$) or the second (MCP: $P > 0.05$) phloem sampling. In the roots and leaves, total GLS concentrations did not differ between the cultivars, but their profiles did. Leaf GLS profiles differed significantly between cultivars (MCP: $P < 0.01$), with indole GLS dominating in Badger Shipper (Fig. 5.2A), similar to what was observed for the phloem GLS. Root profiles also differed significantly between cultivars (MCP: $P < 0.01$). Gluconasturtiin, which was not recorded in Badger Shipper, contributed to this difference. No interaction between soil treatment and cultivar was recorded for GLS profiles in either leaves (MCP: $P > 0.9$) or roots (MCP: $P > 0.05$).

5.3.4.2 Amino acids

At the initial sampling, before aphid infestation, soil treatment did not affect phloem amino acid concentrations ($P > 0.8$ Table S5.1) or profiles (MCP: $P > 0.05$). At the second sampling, *i.e.* after aphid feeding, there was a significant soil treatment effect within Rivera. Compared with control plants, amino acid phloem concentrations were higher in this cultivar on microorganism-treated soils, which also had the highest aphid numbers, and on nematode-treated soils, which in contrast had the lowest aphid numbers (Table S5.1). The differences in amino acid concentrations among treatments after aphid

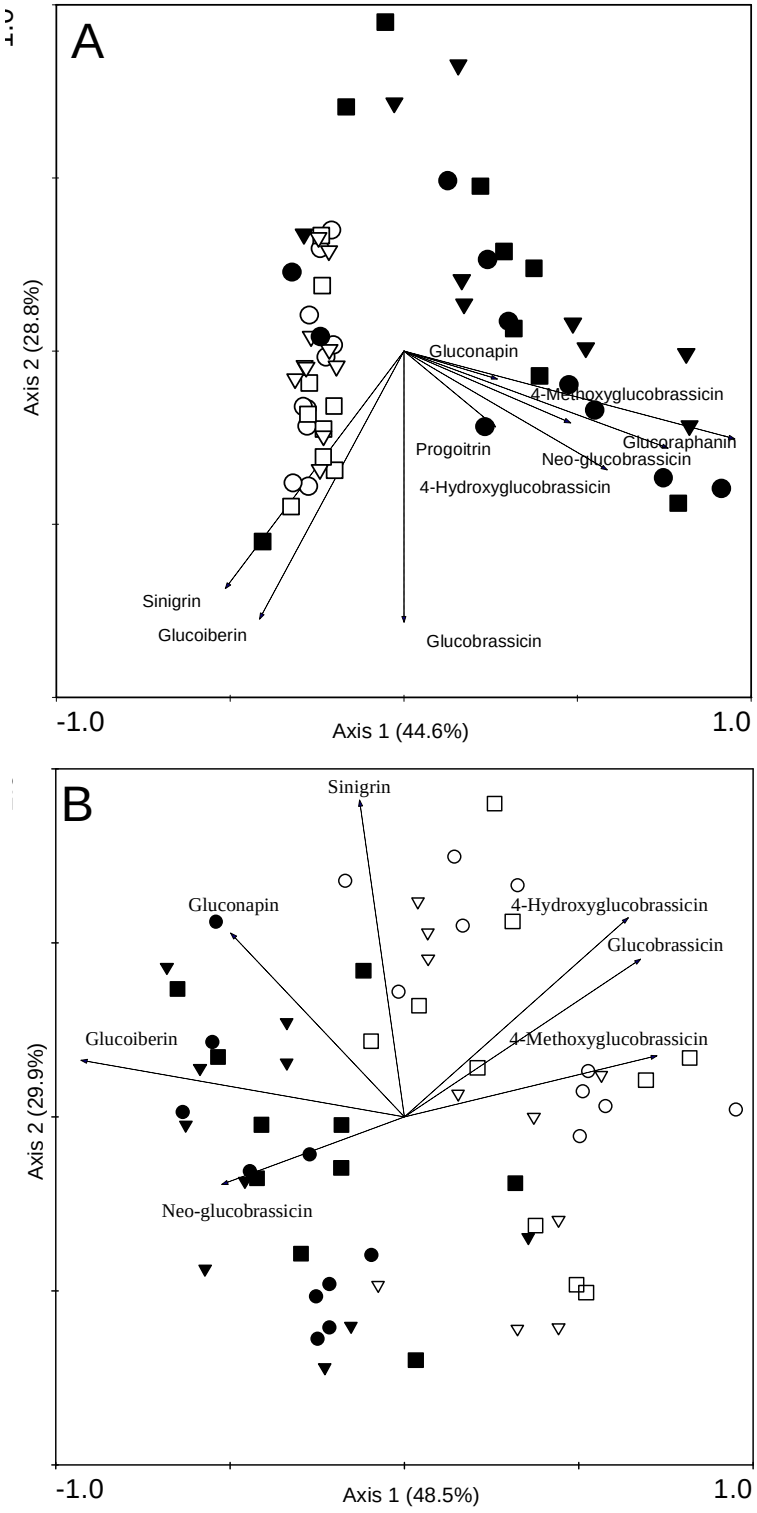


Fig. 5.2 *PCA plot for glucosinolate (GLS) profiles of (A) leaf and (B) aphids. Plants or aphids were raised on soils treated with micro-organisms (circles), untreated control soils (inverted triangles), or nematode-treated soils (squares). White symbols represent the cultivar Rivera and black symbols represent Badger Shipper. Arrows indicate different GLS.*

infestation were accompanied by significant differences in amino acid profiles (MCP: $P < 0.01$).

Amino acid concentration in the phloem initially did not differ between the two cultivars. However, after aphid infestation, Rivera had considerably higher amino acid concentrations than Badger Shipper (Table S5.1). The profiles differed significantly between cultivars before aphid infestation (MCP: $P < 0.001$). Rivera had higher levels of phenylalanine, whereas Badger Shipper had higher concentrations of all other amino acids. After aphid infestation the profiles also differed between the cultivars (MCP: $P < 0.001$), with all amino acids dominating in Rivera.

5.3.5 Aphid chemistry

Total GLS concentrations (two-way ANOVA: $F_{2,58} = 0.67$, $P > 0.1$) and GLS profiles (MCP: $P > 0.5$; Fig. 5.2B) in the aphids were not affected by soil treatment. Total GLS content also did not differ between aphids reared on the different cultivars (two-way ANOVA: $F_{1,58} = 0.48$, $P > 0.1$; Rivera: 16.18 ± 1.20 µmol g^{-1} d. w., Badger Shipper: 17.89 ± 1.68 µmol g^{-1}). GLS profiles, however, varied between the aphids reared on the two cultivars (MCP: $P < 0.01$). In contrast to what was observed in the plants, indole GLS dominated in aphids on Rivera and aliphatic GLS dominated in aphids on Badger Shipper (Fig. 5.2B). There was no significant interaction between soil treatment and cultivar with regard to aphid GLS concentration (two-way ANOVA: $F_{2,54} = 1.07$, $P > 0.1$) or profiles (MCP: $P > 0.5$).

5.3.6 Plant biomass and leaf relative growth rate

Root biomass was higher for control plants than for plants growing in nematode-treated soils, although this contrast was only significant within the Rivera cultivar (Table S5.1). The expansion of the leaves also differed significantly between soil treatments. The growth of the second leaf between weeks 3 and 4 of the experiment was faster in plants on the microorganism and control soils than in the nematode-treated soil, although the contrast was only significant for Badger Shipper.

Plant biomass did not significantly differ between the cultivars before aphid infestation. At the second sampling, after aphid infestation, both root and shoot mass differed significantly between the cultivars. Rivera had a higher root and shoot biomass than Badger Shipper. The relative growth rate of the first and second leaves between weeks 3 and 4 was significantly higher for

Badger Shipper than for Rivera. There was no significant interaction between treatment and cultivar, indicating that cultivars responded similarly in relative growth rate and biomass to the treatments (Table S5.1).

5.4 Discussion

Both manipulation of soil communities and cultivar differences affected aphid population growth on white cabbage. Badger Shipper sustained more aphids than Rivera, irrespective of soil treatment. Microorganisms significantly enhanced aphid population development, whereas nematodes on average reduced aphid population development on both cultivars, although these differences were not statistically significant. Effects of soil treatment on plant parameters and aphid population growth were similar for both cultivars. This indicates that cultivar did not affect the direction (ranking of the soil treatments) or the strength (relative difference between the soil treatments) of the belowground-aboveground interaction. Cultivar also affected fitness parameters of the parasitoids. By contrast, the effects of soil treatment were not detected at the level of parasitoid performance. Overall, these results indicate that effects of belowground organisms on aboveground organisms are consistent across cultivars, *i.e.* independent of the identity of the plant that was mediating the interaction.

Our results are consistent with the observation that, despite the large differences in defensive chemistry between cultivars, their response to belowground organisms with respect to primary and secondary chemistry can be quite similar. In contrast to earlier studies (van Dam and Raaijmakers 2006; Wurst et al. 2006), both cultivars failed to respond to belowground organisms with respect to GLS concentrations or profiles, including those in the phloem that could have affected aphid performance. It will be interesting to see whether the same pattern holds for plants from wild *B. oleracea* populations. The cultivars used in this study are the result of artificial selection and the variation between these plants is well characterized both belowground and aboveground (Poelman et al. 2008b; Poelman et al. 2009b; Kabouw et al. 2010a). Wild *B. oleracea* populations are also known to differ substantially at least in their aboveground GLS composition (Gols et al. 2008b; Newton et al. 2009b), but potentially harbour additional variation in induced responses to belowground organisms that has not been retained in the cultivars. However, similar to cultivated brassicas, two wild populations of *B. vulgaris* showed no induced response in their GLS concentrations to belowground feeding by *D*.

radicum (van Leur et al. 2008), indicating that wild species do not necessarily have to respond differently from cultivated species.

5.4.1 Soil inoculum effect

Aphid population growth was enhanced on plants treated with microorganisms compared with control and nematode-treated plants. This contrasts with results from another study using grasses, in which no differences in aphid numbers were observed between microorganism-treated and control plants (Bezemer et al. 2005). Positive effects of microorganisms on aphid performance can be expected, as belowground (micro)organisms have the ability to influence aboveground insects by mobilizing nutrients and thus improving plant quality (Haase et al. 2008; Eisenhauer et al. 2010; Orwin et al. 2010; Wurst and Forstreuter 2010). If the microorganism effect in our experiment was indeed attributable to improved plant quality it does not seem to be mediated by consistently higher phloem amino acid concentrations or differences in the growth parameters 'relative growth rate' or 'total biomass'.

5.4.2 Effect of cultivar

It is generally known that intraspecific variation, also within the Brassicaceae, can affect aboveground herbivores and higher trophic level organisms (Crutsinger et al. 2008; Kissen et al. 2009; Newton et al. 2009b; Lankau 2011). Our observation that Rivera sustained fewer aphids than Badger Shipper is consistent with other studies (Broekgaarden et al. 2008; Poelman et al. 2009b; Kos et al. 2011a). Aphid population growth generally is dependent on plant traits, such as leaf relative growth rate (Hughes and Bazzaz 2001), and leaf or phloem concentrations of defensive compounds, such as GLS (Kim et al. 2008). Indeed the leaf relative growth rate did differ between cultivars. Moreover, the indole GLS 4-methoxyglucobrassicin and glucobrassicin dominated in the leaves and phloem of Badger Shipper, whereas the aliphatic GLS gluconapin and sinigrin dominated in leaves and phloem of Rivera. Although phloem and leaf total GLS levels were fluctuating (first higher in Badger shipper, later higher in Rivera), it might be the consistent differences in GLS profiles that resulted in different aphid population growth rates. In agreement with our results, we also found positive correlations between indole GLS and *B. brassicae* performance or abundance using the same white cabbage cultivars (Kos et al. 2011a) or several *Arabidopsis thaliana* (L.) Heynh. ecotypes (Kos et al. 2012). However, in other studies mostly negative

correlations between indole GLS and aphid performance have been reported (Cole 1997; Mewis et al. 2005; Kim and Jander 2007; Kim et al. 2008), which is in contrast with the findings of our study. Perhaps specific indole GLS affect aphid performance more strongly than others, and qualitative differences in indole GLS between our study and the literature may have caused the contrasting results.

Soil treatment did not result in different GLS profiles among the aphids, but the cultivars on which they had been reared greatly affected their GLS profiles. Intriguingly, the GLS profiles in the aphids were completely different from those in the phloem: 4-methoxyglucobrassicin and glucobrassicin were recorded in higher concentrations in aphids on Rivera, whereas these dominated in Badger Shipper plants. The incongruence between GLS in the plants and aphids may be due to the ability of *B. brassicae* to selectively sequester GLS, thus regulating GLS composition (Bridges et al. 2002; Kazana et al. 2007; Kos et al. 2011b; 2012). Hence, aphid GLS are not necessarily directly correlated to phloem and leaf GLS levels.

The difference in aphid GLS profiles might have explained why *D. rapae* performed better on aphids reared on Badger Shipper. However, it has been suggested that the performance of *D. rapae* is probably not affected by the sequestered GLS in the host (Le Guigo et al. 2011; Kos et al. 2012). *Brevicoryne brassicae* stores the sequestered GLS in the haemolymph (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008), and contains an endogenous myrosinase that is stored separately from the GLS in the non-flight muscles (Jones et al. 2001; Bridges et al. 2002; Francis et al. 2002). Only when upon damage of the aphid the GLS come together with the aphid myrosinase are toxic breakdown products produced. Tissue-feeding endoparasitoids such as *D. rapae* feed selectively on certain host tissues during most of their larval development (Godfray 1994; Harvey et al. 2000), and possibly due to this feeding strategy, *D. rapae* might prevent the formation of toxic hydrolytic products of GLS in the aphid to a large extent. The better aphid performance on Badger Shipper most likely explains why *D. rapae* performed better on aphids reared on Badger Shipper. Adult mass of *D. rapae* was higher and its development time was shorter when developing on aphids reared on Badger Shipper, two parameters usually associated with beneficial host quality (Bukovinszky et al. 2008). The positive correlation between host performance and parasitoid performance has been reported before (Harvey

2005; Bukovinszky et al. 2008; Kos et al. 2012). In contrast to the difference in adult mass and development time, on Rivera, female sex ratio was higher, also a parameter that is usually associated with better host quality (Tanaka 2009). However, our experiments were not designed to specifically test the effects of host quality on the sex determination by the female, and we do not know what caused the observed difference in sex-ratio between the cultivars.

5.4.3 Conclusion

In conclusion, our study has shown that aphid populations respond to both soil organisms and plant cultivar. However, belowground-aboveground interactions were independent of *Brassica* cultivar. The lack of an intraspecific plant effect on the belowground-aboveground interaction may be the result of rather similar responses of both cultivars in terms of defensive chemistry to the soil treatments. The stimulated aphid population development by microorganisms indicates that plant-animal interactions aboveground may change, depending on soil community composition. However, in our specific case, cultivar had a stronger effect on the aboveground interactions than the composition of the soil community.

5.5 Acknowledgements

The authors thank Remy Hillekens for experimental advice. This research was funded by ERGO grant nr 83806012 of the Netherlands Organization for Scientific Research. Publication 4928 Netherlands Institute of Ecology.

6

Herbivore-mediated effects of glucosinolates on different natural enemies of a specialist aphid

Martine Kos, Benyamin Houshyani, Buddhi B. Achhami, Rafal Wietsma, Rieta Gols, Berhane T. Weldegergis, Patrick Kabouw, Harro J. Bouwmeester, Louise E.M. Vet, Marcel Dicke and Joop J.A. van Loon

Published in a slightly different form in *Journal of Chemical Ecology* (2012), doi: 10.1007/s10886-012-0065-2

Abstract

The cabbage aphid *Brevicoryne brassicae* is a specialist herbivore that sequesters glucosinolates (GLS) from its host plant as a defence against its predators. It is unknown to what extent parasitoids are affected by this sequestration. Our objectives were to investigate herbivore-mediated effects of GLS on the parasitoid wasp *Diaeretiella rapae* and, to allow for comparison with previous studies, the predator *Episyrphus balteatus*.

We reared *B. brassicae* on three ecotypes of *Arabidopsis thaliana* that differ in GLS content and one genetically transformed line with lower phloem concentrations of aliphatic GLS. We tested the performance of the aphid and the performance and behaviour of both natural enemies and correlated this with phloem and aphid GLS concentrations and emission of volatiles.

The performance of *B. brassicae* was positively correlated with the concentrations of both aliphatic and indole GLS in the phloem. The aphid selectively sequestered GLS from the phloem. The GLS concentration in *B. brassicae* was negatively correlated with the performance of the predator, but positively with the performance of the parasitoid, probably because the aphids with the highest GLS concentrations had a higher body weight. Both the predator and the parasitoid preferred the *A. thaliana* ecotype on which its offspring performed best, indicating a positive performance-preference correlation.

Our study shows that there are differential herbivore-mediated effects of GLS on a predator and a parasitoid of a specialist aphid that selectively sequesters GLS from its host plant.

6.1 Introduction

Plants have evolved a wide array of traits that confer resistance to herbivores. For example, toxic secondary metabolites negatively affect the performance of herbivores (Karban and Baldwin 1997; Schoonhoven et al. 2005). Specialist herbivores that are adapted to feeding on plants containing specific secondary metabolites, however, can use these compounds for their own benefit. For example, certain specialists use secondary plant metabolites as oviposition or feeding stimulants (van Loon et al. 1992; Gabrys and Tjallingii 2002). Some specialists concentrate the metabolites actively taken up from the host plant in special tissues or organs. This so-called sequestration can make these herbivores unpalatable for natural enemies (Duffey 1980; Müller 2009).

Brassicaceous plants contain glucosinolates (GLS) that, upon damage by chewing herbivores, become exposed to the plant enzyme myrosinase that hydrolyses GLS, resulting in several toxic volatile compounds such as (iso)thiocyanates and nitriles that negatively affect a wide variety of generalist herbivores (Halkier and Gershenzon 2006; Hopkins et al. 2009). Phloem-feeding herbivores like aphids, however, can ingest GLS from the phloem without bringing these compounds into contact with plant myrosinases (Andreasson et al. 2001). Thus, aphids prevent the formation of toxic hydrolysis products of most GLS (de Vos et al. 2007; Kim and Jander 2007). The cabbage aphid *Brevicoryne brassicae* is a specialist herbivore that uses GLS as feeding stimulants (Gabrys and Tjallingii 2002), and sequesters GLS from its food plant (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008; Kos et al. 2011b). It contains an endogenous myrosinase, which is stored separately from the GLS (Jones et al. 2001; Bridges et al. 2002; Francis et al. 2002). Upon predator attack, the sequestered GLS come in contact with the myrosinase, resulting in the formation of toxic hydrolytic products. Negative effects of this sequestration have been reported for aphid predators, such as ladybird beetles, hoverflies and lacewings (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008; Chaplin-Kramer et al. 2011; Kos et al. 2011b). Most predators kill their prey immediately and feed on multiple prey individuals during their development. Parasitoids, on the other hand, develop inside a single host individual and koinobiont parasitoids allow the host to continue to grow and feed after parasitism (Godfray 1994). Parasitoids are therefore probably differentially affected by sequestration of GLS in *B. brassicae* compared to predators, but this has rarely been investigated. Le Guigo et al. (2011) compared the fitness of the solitary endoparasitoid *Diaeretiella rapae*

when developing in *B. brassicae* aphids that were feeding on host plant species with different foliar GLS concentrations. Parasitoid performance did not correlate with plant GLS concentrations (Le Guigo et al. 2011). In the latter study GLS concentrations in the aphids have not been analysed. It has been shown before that *B. brassicae* sequesters GLS selectively from its food plant (Kos et al. 2011b). It is, therefore, still unknown to what extent *D. rapae* performance is affected by GLS sequestration in *B. brassicae*.

The objective of this study was to investigate herbivore-mediated effects of GLS on the parasitoid *D. rapae* and, to allow for comparison with previous studies, the predator *E. balteatus*. These species represent two different functional groups within the carnivorous insects and are two of the most important natural enemies of *B. brassicae*. To obtain aphids that differ in their sequestered GLS concentrations, we reared them on three ecotypes of *Arabidopsis thaliana* that differ qualitatively and quantitatively in their GLS content (Houshyani et al. 2012). Furthermore, a genetically transformed line was created to produce higher concentrations of foliar aliphatic (methionine-derived) GLS compared to the wild-type plant. We compared the performance of *B. brassicae* on these different ecotypes/lines, analysed the GLS in the phloem and in the aphids feeding on it, and determined the performance of *E. balteatus* and *D. rapae* when feeding on these aphids. The performance of natural enemies can be positively correlated with their preference for a certain herbivore-plant complex (Soler et al. 2007; Gols et al. 2009). Many natural enemies of herbivores use plant volatile organic compounds emitted in response to herbivory to locate prey- or host-infested plants (Dicke and Sabelis 1988; Turlings et al. 1990; Dicke and Baldwin 2010; Mumm and Dicke 2010). Therefore, we also studied parasitoid and predator preference behaviour in response to aphid-induced volatile organic compounds emitted by the different plants ecotypes/lines.

6.2 Materials and methods

6.2.1 Plant material and growth conditions

Three *Arabidopsis thaliana* (L.) Heynh. ecotypes were selected, based on their maximal divergence in metabolite profiles (the qualitative and quantitative composition of the mix of metabolites) (Houshyani et al. 2012). Columbia (Col)-0 was provided by Dr. P. Reymond (Lausanne, Switzerland), Cape Verde Island (Cvi) was obtained from the European *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk/, Cvi = N8580) and Eringsboda (Eri) was

collected in Sweden by members of the Laboratory of Genetics, Wageningen University (Eri-1 = CS22548).

To produce plants with higher foliar levels of aliphatic GLS we over-expressed the transcription factor HAG1/MYB28 in *A. thaliana* ecotype Col-0 (Houshyani et al., in prep., see also Supporting Information Method S6.1). This transcription factor represents a key component in the regulation of aliphatic GLS biosynthesis in *A. thaliana* (Gigolashvili et al. 2007). T2 generation seeds of one successfully transformed line (hereafter named Col-0 -MYB28) were used in the experiments.

Arabidopsis thaliana seeds were surface-sterilized overnight by vapour phase sterilization and inoculated on a growth medium (purified agar 0.8% + 2.2 g L^{-1} 0.5 MS + vitamins; pH 6; containing 30 µg ml^{-1} kanamycin to select transformed seedlings). After four days of stratification at 4 °C, plates were transferred to a growth chamber at 21 ± 2 °C, 50-70% relative humidity (RH) and a 8:16 light:dark (L:D) photoregime, with a light intensity of 200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diameter) containing autoclaved soil (80 °C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times a week and the soil was treated weekly with entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) to control infestation by larvae of sciarid flies. Plants used in the experiments were six to seven weeks old and remained in the vegetative state during the experiments.

6.2.2 Insect rearing

Brevicoryne brassicae L. (Hemiptera: Aphididae) was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus).

Episyrphus balteatus de Geer (Diptera: Syrphidae) pupae were provided by Koppert Biological Systems and kept in gauze cages (67 x 50 x 67 cm). Adults emerging from the pupae were provided with water, a *B. brassicae*-infested *B. oleracea* plant, organic sugar grains and bee-collected pollen provided by Koppert Biological Systems.

Diaeretiella rapae McIntosh (Hymenoptera: Braconidae) was reared in gauze cages (30 x 40 x 60 cm) containing *B. brassicae*-infested *B. oleracea* plants. Wasps were provided with water and honey. The *B. brassicae* and *D. rapae* culture originated from individuals obtained from *B. oleracea* in the

vicinity of Wageningen (The Netherlands) in 2008. All insect species were reared at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime.

6.2.3 GLS and primary metabolites in phloem of aphid-infested plants

After the aphid performance experiment was ended (see below under *Aphid performance*), phloem of aphid-infested plants was collected for chemical analysis. We used 8 mM EDTA, following the procedure described in Kos et al. 2011b. Four fully-grown leaves of each plant were placed with their petiole for 5 min in the EDTA solution to remove any plant chemicals from the incision. Then the leaves were placed for 4 h in a new vial with 200 µl EDTA solution, under dark conditions. Using this method, a small amount of mesophyll fluids was inherently collected as well. Following incubation, the EDTA solution was collected from the vials and each vial was rinsed with 50 µl EDTA solution, resulting in a sample of 250 µl per leaf. The EDTA solution of four plants (16 leaves) was pooled to form one sample of 4 ml, resulting in five replicates per ecotype/line. The leaves were dried at 80 °C for 3 days and weighed on an analytical balance (Mettler-Toledo PM200, Tiel, The Netherlands). Phloem samples were frozen at -80 °C immediately after collection, freeze-dried, and re-suspended in 2 ml 8 mM EDTA. Half of the collected phloem sample was used for GLS extraction, and half for soluble carbohydrate and amino acid extraction. To extract GLS from the phloem, we used the protocol that was described in Kos et al. 2011b. Soluble carbohydrates (50 µl from the one ml sample) and amino acids (50 µl) were extracted and analysed as described previously by Van Dam and Oomen (2008).

6.2.4 Dynamic headspace collection of volatiles from aphid-infested plants

Six-to-seven week-old *A. thaliana* plants were infested with 100 *B. brassicae* nymphs of mixed instars three days prior to the headspace collection. Dynamic headspace collection was carried out in a climate chamber at 20 ± 2 °C. The plants were removed from their pots and the soil was wrapped using aluminium foil. Three plants were placed together in a 2.5 L glass jar. Volatiles were collected by sucking air out of the jar at a rate of 90 ml min^{-1} for 3 h through a stainless steel cartridge (Markes, Llantrisant, UK) containing 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, Illinois, USA). The foliar fresh weight of the plants in each pot was measured after volatile

collection. For each ecotype/line, eight to eleven replicate samples were collected (8 Cvi, 10 Eri, 9 Col-0, 11 Col-0-MYB28).

The headspace samples were analysed using a Thermo Trace Gas Chromatography Ultra (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) quadrupole mass spectrometer (MS) (Method S6.2). The peak area of each compound was expressed per unit plant fresh weight.

6.2.5 Plant morphology and foliar GLS concentrations of uninfested plants

For ten uninfested plants per ecotype/line, we measured foliar biomass and counted the number of trichomes in a 25 mm^2 area in the central part of the abaxial side of the 6^{th} or 7^{th} youngest leaf using a microscope (Leitz Dialux 20 EB, Wetzlar, Germany; magnification 40x).

For foliar GLS analysis, we harvested all leaf material of ten uninfested plants per ecotype/line. Samples were frozen at -80 °C immediately after collection, freeze-dried, weighed (approximately 100 mg) into micro-centrifuge tubes and ground to a fine powder.

GLS were extracted and purified by using a methanol extraction (2004) and GLS were separated using high-performance liquid chromatography (HPLC). For a detailed description of the extraction and analysis of the GLS see Van Dam et al. (2004).

6.2.6 Insect performance

Individual plants with insects were confined to cylindrical plastic containers (height 13 cm; diameter 11 cm) with a gauze lid. The experiments were performed in a climate chamber at 21 ± 2 °C, 50-70% RH and a 8:16 h L:D photoregime for *B. brassicae* and 16:8 L:D for *E. balteatus* and *D. rapae*. The light intensity at plant level was 200 µmol m^{-2} s^{-1} PPFD. Plants were watered once a week.

6.2.6.1 Aphid performance

Several six-week-old plants of each ecotype/line were inoculated with 10 adult aphids per plant. After 24 h, the adult aphids were removed and the produced offspring was allowed to develop for three days until they reached the second instar (L2). Three L2 nymphs were transferred to each of 20 *A. thaliana* plants per ecotype/line, the same ecotype/line as the one on which these nymphs

had been feeding before. Until the adult stage, survival of the nymphs was recorded daily. The fastest developing adult was kept on the plant, while the other adults were removed. Alate (winged) adults (ca. 5% of all adults) were excluded from the experiment as these contain lower concentrations of GLS than apterous (wingless) aphids (Kazana et al. 2007). The development time until first reproduction (=T_d) of the remaining adult was recorded and the adult fresh weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany). The adult was allowed to feed on the plant and produce offspring, and after a certain number of days (equivalent to T_d), the number of offspring (=N) produced by the adult was counted. The estimated intrinsic rate of population increase (r_m) was calculated for each aphid using the formula: r_m = 0.738 x (ln$N$)/T_d (Karley et al. 2002).

6.2.6.2 Aphid GLS concentrations

After the aphid performance experiment was ended, the aphids on the four plants that were used to obtain one phloem sample (as described above for the phloem samples) were removed and pooled into one sample. GLS were extracted similarly to the method used for the leaves.

6.2.6.3 Predator performance

Female *E. balteatus* from the stock rearing were allowed to lay eggs on Brussels sprouts plants infested with *B. brassicae.* After egg hatching, neonate larvae were transferred to *A. thaliana* plants that had been infested by 10 adult *B. brassicae* from the stock rearing one week earlier. The larvae were allowed to develop on the plants until pupation. Pupae were checked once a day for eclosion of adults. Survival, larva-to-adult development time, sex and adult dry weight were determined. Newly eclosed adults were frozen to death, dried to constant weight at 80 ˚C for 3 days and then weighed on a microbalance. We determined the performance of 35 larvae per *A. thaliana* ecotype/line, one larva per plant.

6.2.6.4 Parasitoid performance

Aphid mummies containing a *D. rapae* pupa were collected from the stock rearing and reared until adult parasitoid eclosion. Adult parasitoids were provided with water and honey, allowed to mate and used for parasitisation when they were 2-4 days old. Second instar (three day old) *B. brassicae* nymphs that had been feeding on one of the *A. thaliana* ecotypes/lines were

exposed individually to mated female parasitoids on an aphid-infested leaf until parasitisation was observed (*i.e.* when the female inserted her ovipositor in the nymph). Four parasitized nymphs were transferred to one *A. thaliana* plant of the same ecotype/line as the one on which these aphids had been feeding before. In total we tested 22 plants per *A. thaliana* ecotype/line. Mummies were collected from the plants and after eclosion, parasitoid sex was determined, and egg-to-adult development time and adult dry weight were measured, as described for the predator. The percentage of successful parasitism of *B. brassicae* by *D. rapae* was calculated per plant by dividing the number of *D. rapae* adults by the total number of *B. brassicae* nymphs that survived (either until the adult stage or until *D. rapae* eclosion) on each plant.

6.2.7 Predator and parasitoid preference

The preference of predators and parasitoids for volatiles from an ecotype/line was investigated in two-choice bioassays. We tested the ecotypes against each other, and Col-0-MYB28 against Col-0. Plants were treated similarly as described above under *Dynamic headspace collection of volatiles from aphid-infested plants*.

6.2.7.1 Predator oviposition preference

Mated female hoverflies from the stock rearing were used in the behavioural assays when they were two to three weeks old. Females were transferred to a plastic cage (30 x 30 x 30 cm) containing one aphid-infested plant of two different ecotypes/lines, and 10% sugar solution. Females were allowed to oviposit on the plants for 24 h. The number of eggs deposited on each plant was counted. Replicates with females that did not lay any eggs were eliminated from the analysis. For each plant combination, at least 22 replicates with ovipositing females were obtained.

6.2.7.2 Parasitoid preference for aphid-induced plant volatiles

Parasitoid behaviour was assessed in a Y-tube olfactometer in a climatized room at 22 ± 2 °C as described by Bukovinszky et al. (2005). Compressed air was filtered over charcoal and split into two air streams each at a flow of 2 L min^{-1}. Each air stream was led through a 5 L glass jar that contained four plants of one of the two ecotypes/lines of a test combination. Each air stream was then led into one of the two arms of the Y-tube. The olfactometer was illuminated from above using artificial light at an intensity of 60 µmol m^{-2} s^{-1}

PPFD.

Naïve, mated two-day-old *D. rapae* females were allowed to oviposit for one h in aphids feeding on one of the two ecotypes/lines of a combination (equally divided among the tested wasps) to increase their motivation to search for a host. Experienced wasps were individually released at the base of the Y-tube and their preference for one of both odour sources was recorded. A choice was recorded when a wasp crossed a finish line drawn one cm before the end of each arm, and did not return to the junction within 15 s. Wasps that did not make a choice within 15 min were considered as non -responsive and were omitted from the statistical analysis. Four or five new sets of plants were used for each test combination. For every new set of plants, 20 wasps were tested. After every ten wasps, the position of the odour sources was exchanged to compensate for any asymmetry in the set-up.

6.2.8 Statistical analysis

Analyses were performed in SPSS for Windows (18th edition, Chicago, Illinois, USA), unless indicated otherwise. If variables were log-transformed to obtain normality and equal variance, this is indicated in the relevant table of the *Results* section. To test the effect on the continuous variables, such as development time and body weight, we used ANOVA followed by post-hoc Tukey-tests for pair-wise ecotype comparisons and Student's t-tests for comparisons between Col-0 and Col-0-MYB28. If assumptions on normality and equal variance were violated, Kruskal-Wallis tests with post-hoc Mann-Whitney U tests with a Holm's sequential Bonferroni correction were used for pair-wise ecotype comparisons, and Mann-Whitney U tests for comparisons between Col-0 and Col-0-MYB28. Survival and the percentage of successful parasitism were calculated per plant (plant was used as the experimental unit), and differences in these variables among ecotypes and between Col-0 and Col-0-MYB28 were analysed by logistic regression in GenStat (13^{h} edition, VSN International, UK). If over-dispersion was observed, the data were corrected for this by using estimated dispersion instead of fixed dispersion. T-probabilities were calculated to test pair-wise differences between means.

To test whether an equal number of predator eggs was laid on each ecotype/line in a test combination, Wilcoxon's matched-pairs signed-rank tests were used. To test whether an equal number of parasitoid wasps chose either ecotype/line in a test combination in the Y-tube olfactometer, Chi-square tests

were used. Effects of parasitoid experience on the preference of the wasps was tested by logistic regression in GenStat.

To test if there were differences in volatile profiles and aphid GLS profiles among the ecotypes and between Col-0 and Col-0-MYB28, we used multivariate discriminant analysis Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12^{th} edition, Umetrics, Umeå, Sweden) (Eriksson et al. 2006). Partial Least Squares Projections to Latent Structures (PLS) in SIMCA-P, a multivariate method for regression analysis, was used to test the relationship between a) metabolite profiles in the phloem and performance of aphids feeding on those plants, and b) the GLS profile in the phloem and in the aphids feeding on those plants. For the latter PLS-analysis, only the GLS compounds that were found in both the phloem and the aphids, as well as the total, total aliphatic and total indole GLS, were included. To pre-process data, metabolite concentrations were log-transformed, mean-centred and scaled to unit variance. To test whether concentrations of individual GLS compounds or classes in the phloem and aphids were correlated we used Spearman's correlation test. PLS analyses of the relationships between aphid GLS concentrations and predator/parasitoid performance could not be performed, as we measured these variables in separate experiments. Note that in the *Results* section 'aliphatic GLS' refers to the total of all aliphatic GLS compounds that were detected, 'indole GLS' refers to the total of all indole GLS compounds, and 'total GLS' refers to the total of all GLS compounds (aliphatic and indole GLS combined).

6.3 Results

6.3.1 GLS and primary metabolites in phloem of aphid-infested plants

Ecotype effect: Total, aliphatic and indole GLS concentrations in the phloem of aphid-infested plants differed among the ecotypes (Kruskal-Wallis H, *d.f.* = 2, total: $\chi^2 = 6.48$, $P = 0.039$; aliphatic: $\chi^2 = 8.07$, $P = 0.018$; indole: $\chi^2 = 10.82$, $P = 0.004$), due to both qualitative and quantitative differences (Table 6.1). Phloem of Cvi had the highest total and aliphatic GLS concentrations, whereas phloem of Eri plants had the highest indole GLS concentrations. Phloem of Col-0 plants had the lowest concentration of all classes of GLS (Table 6.1). The ecotypes did not differ in total concentrations of carbohydrates and amino acids in the phloem (ANOVA, $P > 0.05$ for both analyses), although there were small qualitative and quantitative differences in the concentrations of the individual compounds (Table 6.1).

Table 6.1 Mean (± SE) concentrations of metabolites in the phloem of aphid-infested plants of three *Arabidopsis thaliana* ecotypes and the transformed Col-0-MYB28 line, and in *Brevicoryne brassicae* aphids reared on these plants

			Arabidopsis thaliana ecotype			Transformed
	Metabolite		**Cvi**	**Eri**	**Col-0**	**Col-0-MYB28**
Phloem	Carbo-hydrates[a]	sorbitol	0.002 ± 0.001	0.005 ± 0.001	0.002 ± 0.002	0.002 ± 0.002
		mannitol	0.008 ± 0.001	0.008 ± 0.002	0.010 ± 0.002	0.014 ± 0.003
		trehalose	0.010 ± 0.009	0.001 ± 0.001	0.002 ± 0.001	0.013 ± 0.008
		glucose	0.392 ± 0.069	0.149 ± 0.020	0.156 ± 0.010	0.216 ± 0.014
		fructose	0.321 ± 0.060	0.097 ± 0.015	0.112 ± 0.008	0.166 ± 0.012
		sucrose	0.988 ± 0.191	2.328 ± 0.305	1.892 ± 0.142	1.949 ± 0.147
		raffinose	0	0.015 ± 0.006	0	0
		Total carbohydrates	1.721 ± 0.312**a**	2.604 ± 0.340**a**	2.175 ± 0.139**a**	2.318 ± 0.148**ns**
	Amino acids[a]	arginine	0.095 ± 0.017	0.080 ± 0.011	0.136 ± 0.012	0.157 ± 0.014
		lysine	0.016 ± 0.006	0.024 ± 0.005	0.040 ± 0.006	0.053 ± 0.008
		glutamine	2.474 ± 0.370	2.348 ± 0.292	3.671 ± 0.375	3.552 ± 0.363
		asparagine	0.139 ± 0.022	0.065 ± 0.010	0.032 ± 0.016	0.027 ± 0.014
		alanine	0.026 ± 0.013	0.036 ± 0.005	0.070 ± 0.008	0.099 ± 0.011
		threonine	0.833 ± 0.148	0.313 ± 0.042	0.472 ± 0.037	0.610 ± 0.048
		valine	0.018 ± 0.008	0.036 ± 0.006	0.065 ± 0.011	0.090 ± 0.015
		serine	0.081 ± 0.017	0.065 ± 0.011	0.121 ± 0.013	0.077 ± 0.008
		leucine	0.010 ± 0.006	0	0.008 ± 0.005	0.009 ± 0.006
		methionine	0.001 ± 0.001	0	0.002 ± 0.002	0.001 ± 0.001
		histidine	0.004 ± 0.004	0.012 ± 0.001	0.021 ± 0.003	0.095 ± 0.014
		phenylalanine	0.017 ± 0.004	0.014 ± 0.002	0.033 ± 0.004	0.034 ± 0.004
		glutamate	0.066 ± 0.001	0.040 ± 0.004	0.098 ± 0.009	0.114 ± 0.010
		aspartate	0.058 ± 0.012	0.047 ± 0.009	0.113 ± 0.016	0.091 ± 0.013
		tyrosine	0.007 ± 0.002	0.009 ± 0.001	0.015 ± 0.004	0.020 ± 0.005
		Total amino acids[b]	3.844 ± 0.592**a**	3.088 ± 0.390**a**	4.897 ± 0.470**a**	5.028 ± 0.480**ns**
	GLS[c,d]	epiprogoitrin	1.958 ± 1.199	0	0	0
		sinigrin	1.208 ± 0.803	1.028 ± 1.028	0	0

		gluconapin	1.030 ± 0.699	0	0	0
		glucoiberverin	1.694 ± 0.237	0.384 ± 0.250	0.735 ± 0.051	0.147 ± 0.010
		Total aliphatic GLS	5.889 ± 1.860**b**	2.426 ± 1.258**a**	0.915 ± 0.186**a**	0.147 ± 0.010*
		glucobrassicin	0.348 ± 0.100	0.705 ± 0.069	0.302 ± 0.068	0.248 ± 0.055
		4-methoxyglucobrassicin	0.507 ± 0.073	0.805 ± 0.064	0.309 ± 0.054	0.297 ± 0.052
		Total indole GLS	0.855 ± 0.055**a**	1.511 ± 0.117**b**	0.611 ± 0.102**a**	0.544 ± 0.090**ns**
		Total GLS	6.744 ± 1.851**b**	3.936 ± 1.287**ab**	1.526 ± 0.253**a**	0.691 ± 0.086*
Aphid	GLS[d,e]	glucoiberin	0	30.04 ± 17.38	5.61 ± 3.54	2.36 ± 1.49
		glucoraphanin	2.13 ± 0.52	5.53 ± 2.77	70.08 ± 35.61	46.96 ± 23.86
		sinigrin	76.59 ± 10.86	7.49 ± 1.35	7.27 ± 2.25	3.05 ± 0.95
		gluconapoleiferin	0.57 ± 0.19	0.09 ± 0.09	2.76 ± 1.20	1.16 ± 0.50
		gluconapin	272.87 ± 49.87	1.08 ± 0.86	2.47 ± 1.18	1.04 ± 0.50
		glucoiberverin	0.16 ± 0.07	2.19 ± 1.22	0	0
		glucoerucin	0.25 ± 0.12	1.66 ± 0.88	3.16 ± 1.31	1.32 ± 0.55
		3-hydroxypropyl	0.42 ± 0.08	99.01 ± 43.23	5.87 ± 3.78	2.47 ± 1.59
		glucosiberin	16.37 ± 2.71	3.72 ± 1.45	6.68 ± 2.67	1.94 ± 0.78
		glucohirsutin	210.25 ± 37.20	118.69 ± 45.04	34.49 ± 13.11	2.07 ± 0.79
		Total aliphatic GLS	579.61 ± 98.59**b**	269.50 ± 111.37**ab**	138.40 ± 58.95**a**	62.36 ± 28.58**ns**
		glucobrassicin	29.63 ± 8.45	30.14 ± 18.29	7.20 ± 1.87	9.36 ± 2.43
		4-hydroxyglucobrassicin	0.44 ± 0.18	1.11 ± 0.64	0.19 ± 0.19	0.05 ± 0.05
		4-methoxyglucobrassicin	0.85 ± 0.11	2.30 ± 0.36	3.16 ± 0.40	2.75 ± 0.35
		neo-glucobrassicin	0.05 ± 0.04	0.08 ± 0.05	0	0
		Total indole GLS	30.98 ± 8.71**a**	33.63 ± 19.20**a**	10.54 ± 2.14**a**	12.16 ± 2.60**ns**
		Total GLS	610.59 ± 104.11**b**	303.13 ± 130.08**ab**	148.94 ± 60.76**a**	74.52 ± 30.89**ns**

*[a] µmol g^{-1} dry weight leaf. [b] Parameter was log-transformed in statistical analysis to obtain normality. [c] nmol g^{-1} dry weight leaf. [d] Glucosinolates (GLS) are grouped according to their biosynthetic origin. [e] µmol g^{-1} dry weight aphids. Statistical tests were performed only for the total carbohydrate, amino acid and GLS concentrations, not for individual compounds. Different letters denote differences in means among the three ecotypes as analysed by Mann-Whitney U-tests with sequential Bonferroni correction (for GLS) or ANOVA and post-hoc Tukey tests (for carbohydrates and amino acids). * denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analysed by Mann-Whitney U-tests (for GLS) or Student's t-tests (for carbohydrates and amino acids). For every sample, phloem or aphids collected from four plants were pooled. See Table S6.2 for the scientific names of all GLS compounds.*

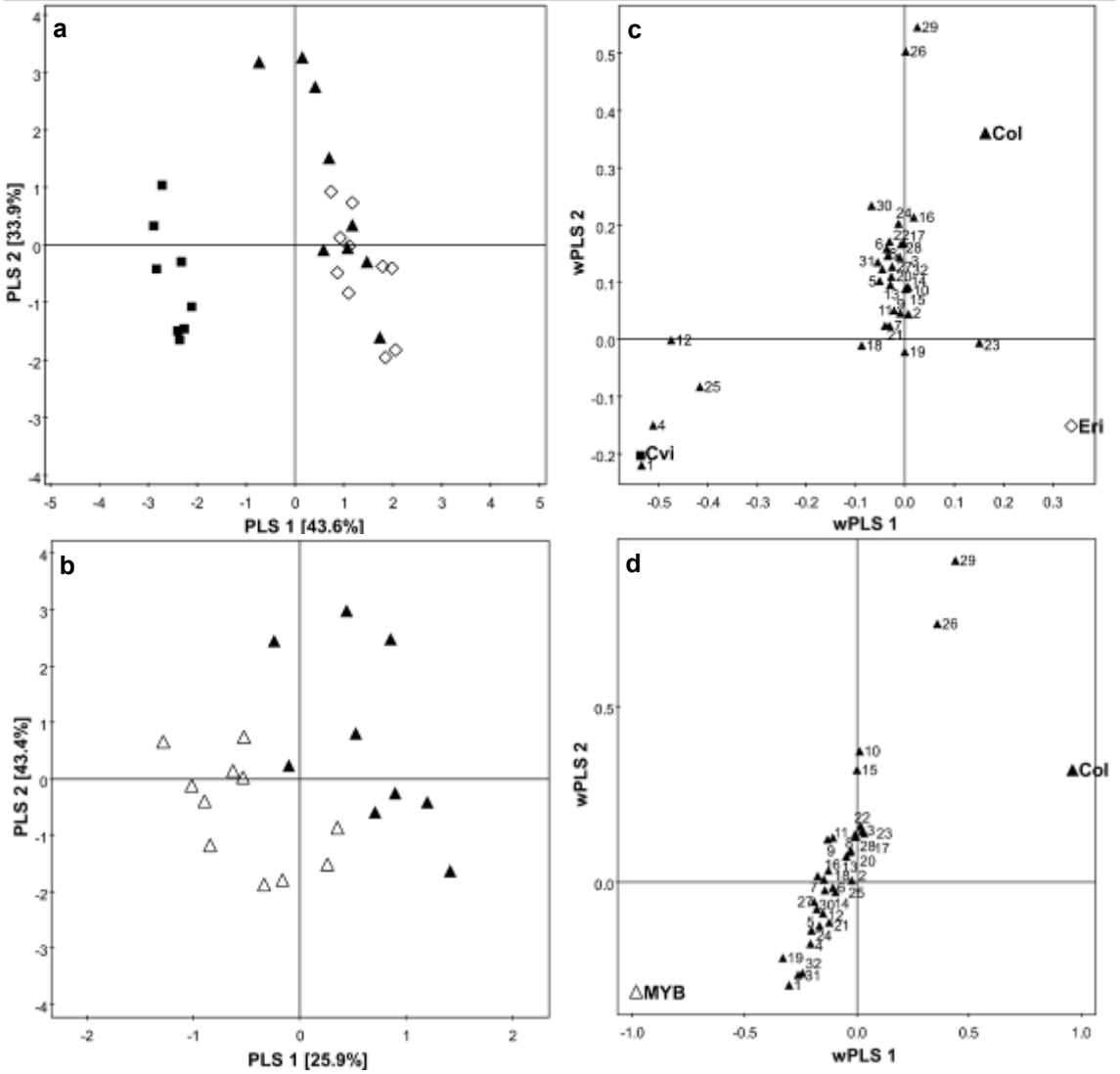


Fig. 6.1 *PLS-DA score and loading plots of the first two components based on the volatile emission of aphid-infested plants of three Arabidopsis thaliana ecotypes (**a** and **b**) and of ecotype Col-0 and the transformed Col-0-MYB28 (**c** and **d**). Plant ecotypes investigated are Cvi (filled boxes), Eri (open diamonds) and Col-0 (filled triangles); the transformed line is Col-0-MYB28 (open triangles). The score plots (**a** and **c**) show the distinction in volatile profiles of the ecotypes/lines. In brackets the percentage of variation explained is indicated. The loading plots (**b** and **d**) show the contribution of the volatile compounds to the discrimination among the ecotypes/lines. Volatile compounds: 1 = 3-butene nitrile, 2 = 2-pentanone, 3 = 4-methyl-2-pentanone, 4 = 3-methyl-3-butene nitrile, 5 = 1-pentanol, 6 = 2-hexanone, 7 = butyl acetate, 8 = 2-pentyl acetate, 9 = styrene, 10 = cumene, 11 = isocumene, 12 = 3-butenyl isothiocyanate, 13 = hemimellitene, 14 = p-cymene, 15 = limonene, 16 = o-cresol, 17 = m-cymene, 18 = ɣ-terpinene, 19 = linalool, 20 = cis-limonene-1,2-epoxide, 21 = menthol, 22 = 1-methylene-1H-indene, 23 = methyl salicylate, 24 = diethyl-2-methylene succinate, 25 = cyclosativene, 26 = daucene, 27 = ɣ-elemene, 28 = longifolene, 29 = δ-selinene, 30 = 6-methyl-α-ionone, 31 = lilial, 32 = farnesylacetaldehyde. See Table S6.3 for additional information about the volatile compounds detected in this study.*

Over-expression effect: The phloem of aphid-infested Col-0-MYB28 plants had lower concentrations of total and aliphatic GLS than Col-0 plants, and similar concentrations of indole GLS (Mann-Whitney U-test: total: $U < 0.001$, $P = 0.008$; aliphatic: $U < 0.001$, $P = 0.008$; indole: $U = 7.00$, $P = 0.310$; Table 6.1). This was unexpected, because foliar tissue of Col-0-MYB28 plants had higher concentrations of aliphatic GLS than Col-0 plants (Table S6.1). Total concentrations of carbohydrates and amino acids in the phloem did not differ between Col-0 and Col-0-MYB28 plants (Table 6.1).

6.3.2 Dynamic headspace collection of aphid-infested plants

Ecotype effect: The three *A. thaliana* ecotypes differed in the volatile profile of aphid-infested plants (4 PLS-DA principal components, $R_2X_{cum} = 0.906$, $R_2Y_{cum} = 0.836$, $Q_{2cum} = 0.682$). The volatile profile of Cvi was high in breakdown products of GLS such as 3-butenyl isothiocyanate, 3-butene nitrile and 3-methyl-3-butene nitrile. Col-0 plants emitted larger amounts of the sesquiterpenes daucene and δ-selinene, whereas the headspace of Eri plants was high in the ester methyl salicylate (Fig. 6.1a, b).

Over-expression effect: The volatile profiles of Col-0 and Col-0-MYB28 plants could be separated by PLS-DA (4 PLS-DA principal components, $R_2X_{cum} = 0.861$, $R_2Y_{cum} = 0.932$, $Q_{2cum} = 0.612$). Col-0-MYB28 emitted, in addition to other compounds, relatively large amounts of GLS breakdown products such as 3-butene nitrile and 3-methyl-3-butene nitrile (Fig. 6.1c, d).

6.3.3 Plant morphology

Ecotype effect: Eri plants had a higher biomass than plants of the other ecotypes. Cvi plants had the highest and Eri plants the lowest trichome density (Table S6.1). Over-expression effect: There was no difference in biomass or trichome density between Col-0 and Col-0-MYB28 plants (Table S6.1).

6.3.4 Aphid performance

Ecotype effect: Aphid survival differed among the ecotypes, although this difference was only marginally significant (logistic regression, $P = 0.051$, Table 6.2). Aphid development time, adult weight, number of offspring and estimated intrinsic rate of population increase (r_m) differed among the ecotypes (ANOVA, respectively $F_{2,57} = 15.10$, $P < 0.001$; $F_{2,57} = 30.24$, $P < 0.001$; $F_{2,57} = 18.62$, $P < 0.001$; $F_{2,57} = 45.66$, $P < 0.001$). All aphid performance parameters were higher (development time shorter) on Cvi

plants than on plants of the other ecotypes (Table 6.2).

Over-expression effect: There was no difference in any of the measured performance parameters of *B. brassicae* between Col-0 and Col-0-MYB28 plants ($P > 0.05$ for every comparison, Table 6.2).

All measured aphid performance parameters were significantly positively correlated (development time inversely correlated) with total and aliphatic GLS and several carbohydrates (4 PLS principal components, $R_2X_{cum} = 0.757$, $R_2Y_{cum} = 0.735$, $Q_{2cum} = 0.343$; Fig. 6.2). The aphid performance parameters were, to a lesser extent, also positively correlated with indole GLS, but this was only significant for development time (inversely correlated). The aphid performance parameters were negatively (although mostly not significantly) correlated with several amino acids, sucrose and total carbohydrates.

6.3.5 Aphid GLS concentrations

Ecotype effect: Aphids reared on the different ecotypes differed in total and aliphatic GLS concentrations (Kruskal-Wallis H, *d.f.* = 2, total: $\chi^2 = 6.98$, $P = 0.031$; aliphatic: $\chi^2 = 6.98$, $P = 0.031$), due to both qualitative and quantitative differences (Table 6.1). Aphids reared on Cvi plants contained the highest and aphids reared on Col-0 plants the lowest total and aliphatic GLS concentrations (Table 6.1). There were no differences in indole GLS among aphids reared on the different ecotypes (Kruskal-Wallis H, $P > 0.05$).

Over-expression effect: Aphids reared on Col-0-MYB28 plants had similar concentrations of total, aliphatic and indole GLS as aphids reared on Col-0 plants (Mann-Whitney U, $P > 0.05$ for every analysis; Table 6.1).

6.3.6 Correlations between GLS profiles in phloem and in *B. brassicae* aphids

In both the univariate Spearman's correlation tests, as well as the multivariate PLS model, the concentrations of most of the GLS compounds or classes in the aphids were positively correlated with their concentrations in the phloem. The positive correlations were, however, only significant for total and aliphatic GLS (PLS model: 1 PLS principal component, $R_2X = 0.486$, $R_2Y = 0.373$, $Q_2 = 0.280$; Spearman's correlation: gluconapin: $r_s = 0.53$; $P = 0.015$; aliphatic: $r_s = 0.72$; $P < 0.001$; total: $r_s = 0.82$; $P < 0.001$). Interestingly, the concentration of the indole 4-methoxyglucobrassicin was negatively correlated, although not significantly, between aphids and the phloem they were feeding on (Fig. 6.3;

Table 6.2 Mean (± SE) performance characteristics of *Brevicoryne brassicae*, *Episyrphus balteatus* and *Diaeretiella rapae* reared on three *Arabidopsis thaliana* ecotypes and the transformed Col-0-MYB28 line

		A. thaliana ecotype[a]			Transformed line[b]
Species	**Performance parameter**	**Cvi**	**Eri**	**Col-0**	**Col-0-MYB28**
B. brassicae	Survival until adult stage (%)[c,d]	98 **a**	92 **a**	90 **a**	95 **ns**
	Development time until first reproduction in days (T_d)[e,f]	8.0 ± 0.1 **a**	8.4 ± 0.1 **a**	9.1 ± 0.2 **b**	9.2 ± 0.2 **ns**
	Adult fresh weight in mg[f]	0.635 ± 0.025 **b**	0.471 ± 0.020 **a**	0.408 ± 0.018 **a**	0.393 ± 0.015 **ns**
	No. of offspring (N) in time period equivalent to T_d[e,f]	33.2 ± 1.5 **b**	22.2 ± 1.2 **a**	22.2 ± 1.7 **a**	19.2 ± 1.2 **ns**
	Estimated intrinsic rate of population increase (r_m)[f]	0.322 ± 0.005 **c**	0.270 ± 0.006 **b**	0.250 ± 0.006 **a**	0.236 ± 0.006 **ns**
E. balteatus	Survival until adult stage (%)[c]	17 **a**	40 **a**	26 **a**	20 **ns**
	Larva-to-adult development time in days[e,f]	21.5 ± 1.1 **c**	16.3 ± 0.3 **a**	18.4 ± 0.6 **b**	16.7 ± 0.6 **ns**
	Adult dry weight in mg[e,f]	3.30 ± 0.53 **a**	2.74 ± 0.19 **a**	2.72 ± 0.16 **a**	3.25 ± 0.40 **ns**
D. rapae	Successful parasitism (%)[c,d]	73 ± 5 **a**	76 ± 5 **a**	57 ± 7 **a**	64 ± 7 **ns**
	Larva-to-adult development time in days[e,f]	11.1 ± 0.1 **a**	11.2 ± 0.1 **a**	11.1 ± 0.1 **a**	11.3 ± 0.1 **ns**
	Adult dry weight in mg[f]	0.068 ± 0.002 **b**	0.058 ± 0.002 **a**	0.059 ± 0.002 **a**	0.055 ± 0.002 **ns**

[a] Different letters denote differences in means among the three ecotypes. [b] ns denotes no significant difference between Col-0 and Col-0-MYB28. [c] Analysed by logistic regression and post-hoc T-probability tests. [d] Performance parameter was averaged per plant before statistical analysis. [e] Performance parameter was log-transformed in statistical analysis to obtain normality. [f] Analysed by ANOVA and post-hoc Tukey tests (for the ecotypes), or Student's t-test (for the wild-type and transformed Col-0 line).

Spearman's correlation, $P > 0.05$).

The contribution of indole GLS to the total concentration of GLS was lower in aphids than in the phloem of the aphid-infested plants (10% indole GLS in aphids compared to 42% indole GLS in the phloem, as averaged over all four ecotypes/lines; see also Table 6.1). Furthermore, the ratio of the indole compounds was different in the aphids than in the phloem: in the phloem the concentration of 4-methoxyglucobrassicin was higher than the concentration of glucobrassicin, whereas this was reverse in the aphids (Table 6.1).

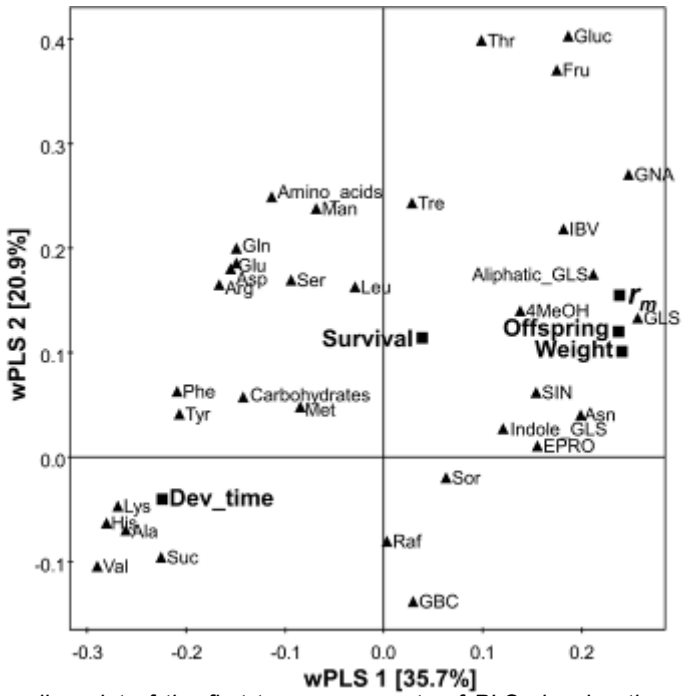


Fig. 6.2 *Loading plot of the first two components of PLS showing the contribution of each individual compound or compound class measured in the phloem of Arabidopsis thaliana, i.e. glucosinolates (GLS), carbohydrates and amino acids, to the performance of the aphid Brevicoryne brassicae in terms of survival, development time, adult weight, number of offspring and estimated intrinsic rate of population increase (r_m). In brackets the percentage of variation explained is indicated. Compound abbreviations: Aliphatic GLS: EPRO = epiprogoitrin, GNA = gluconapin, IBV = glucoiberverin, SIN = sinigrin. Indole GLS: GBC = glucobrassicin, 4MeOH = 4-methoxyglucobrassicin. See Table S6.2 for the scientific names of all GLS compounds. Carbohydrates: Fru = fructose, Gluc = glucose, Man = mannitol, Raf = raffinose. Sor = sorbitol, Suc = sucrose, Tre = trehalose. Amino acids: Ala = alanine, Arg = arginine, Asn = asparagine, Asp = aspartate, Glu = glutamate, Gln = glutamine, His = histidine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine*

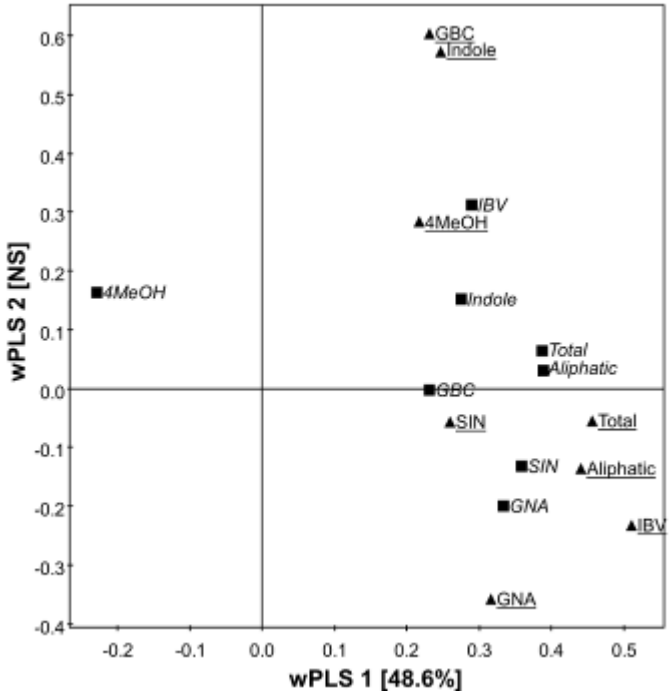


Fig. 6.3 *Loading plot of the first two components of PLS showing the relationship of the concentration of each glucosinolate (GLS) compound or class (aliphatic, indole and total) in the phloem of Arabidopsis thaliana (squares, label in italics) with the concentration of these compounds or classes in the aphid Brevicoryne brassicae (triangles, label underlined) feeding on the phloem. In brackets the percentage of variation explained is indicated. Note that only the first component is significant according to the multivariate model, whereas two components were included to enhance the clarity of the figure. Compound abbreviations: Aliphatic GLS: GNA = gluconapin, IBV = glucoiberverin, SIN = sinigrin. Indole GLS: GBC = glucobrassicin, 4MeOH = 4-methoxyglucobrassicin. See Table S6.2 for the scientific names of all GLS compounds*

6.3.7 Predator performance

Ecotype effect: Survival of *E. balteatus* until the adult stage did not differ among the ecotypes (logistic regression, $P > 0.05$, Table 6.2). Larva-to-adult development time of the hoverflies was affected by plant ecotype and hoverfly sex (ANOVA, ecotype: $F_{2,23} = 27.11$, $P < 0.001$; sex: $F_{1,23} = 8.10$, $P = 0.009$). Hoverflies developed slowest on aphids fed on Cvi plants and fastest on aphids fed on Eri plants (Table 6.2). Averaged over the ecotypes, male hoverflies took longer (18.7 ± 0.8 days) to develop into adults than females (17.3 ± 0.5 days). However, the difference in development time between

males and females was only significant on Cvi, and not on Col-0 and Eri, resulting in a significant interaction between ecotype and sex ($F_{2,23}$ = 4.65, P = 0.020). Adult dry weight was affected by hoverfly sex (ANOVA, $F_{1,23}$ = 6.63, P = 0.017), as male hoverflies (3.18 ± 0.23 mg) were heavier than females (2.49 ± 0.13 mg), but not by plant ecotype (Table 6.2) or the interaction between ecotype and sex (ANOVA, P > 0.05 for both analyses).

Over-expression effect: There was no difference in survival, development time and adult dry weight of *E. balteatus* between Col-0 and Col -0-MYB28 (P > 0.05 for all parameters; Table 6.2).

6.3.8 Parasitoid performance

Ecotype effect: Plant ecotype did not affect the percentage of successful parasitism of *B. brassicae* by *D. rapae* (logistic regression, P > 0.05), nor did it affect egg-to-adult development time (ANOVA, P > 0.05; Table 6.2). Only adult dry weight was affected by plant ecotype (ANOVA, ecotype: $F_{2,169}$ = 10.16, P < 0.001). Adult dry weight was higher on Cvi plants than on plants of the other ecotypes (Table 6.2). There was no effect of parasitoid sex or the interaction between ecotype and sex for any of the performance parameters (P > 0.05 for all parameters).

Over-expression effect: There was no difference in the percentage of successful parasitism, development time and adult dry weight of *D. rapae* between Col-0 and Col-0-MYB28 (P > 0.05 for all parameters; Table 6.2).

6.3.9 Predator oviposition preference

Female *E. balteatus* preferred to oviposit on aphid-infested Eri plants over aphid-infested Col-0 and Cvi plants, and aphid-infested Col-0 plants over aphid-infested Cvi plants (Wilcoxon's matched-pairs signed-rank test: Eri vs Col-0: Z = 2.63, n = 27, P = 0.008; Eri vs Cvi: Z = 3.44, n = 22, P = 0.001; Col-0 vs Cvi: Z = 2.12, n = 29, P = 0.034). Females did not differentiate between aphid-infested plants of Col-0 and Col-0-MYB28 (Wilcoxon's matched-pairs signed-rank test, P > 0.05; Fig. 6.4).

6.3.10 Parasitoid preference for aphid-induced plant volatiles

Female *D. rapae* preferred volatiles from aphid-infested Cvi plants over volatiles from aphid-infested Col-0 plants (Chi-square, χ^2 = 5.69, P = 0.017). Females neither differentiated between volatiles from any of the other ecotype combinations, nor between Col-0 and Col-0-MYB28 (Chi-square, P > 0.05 for

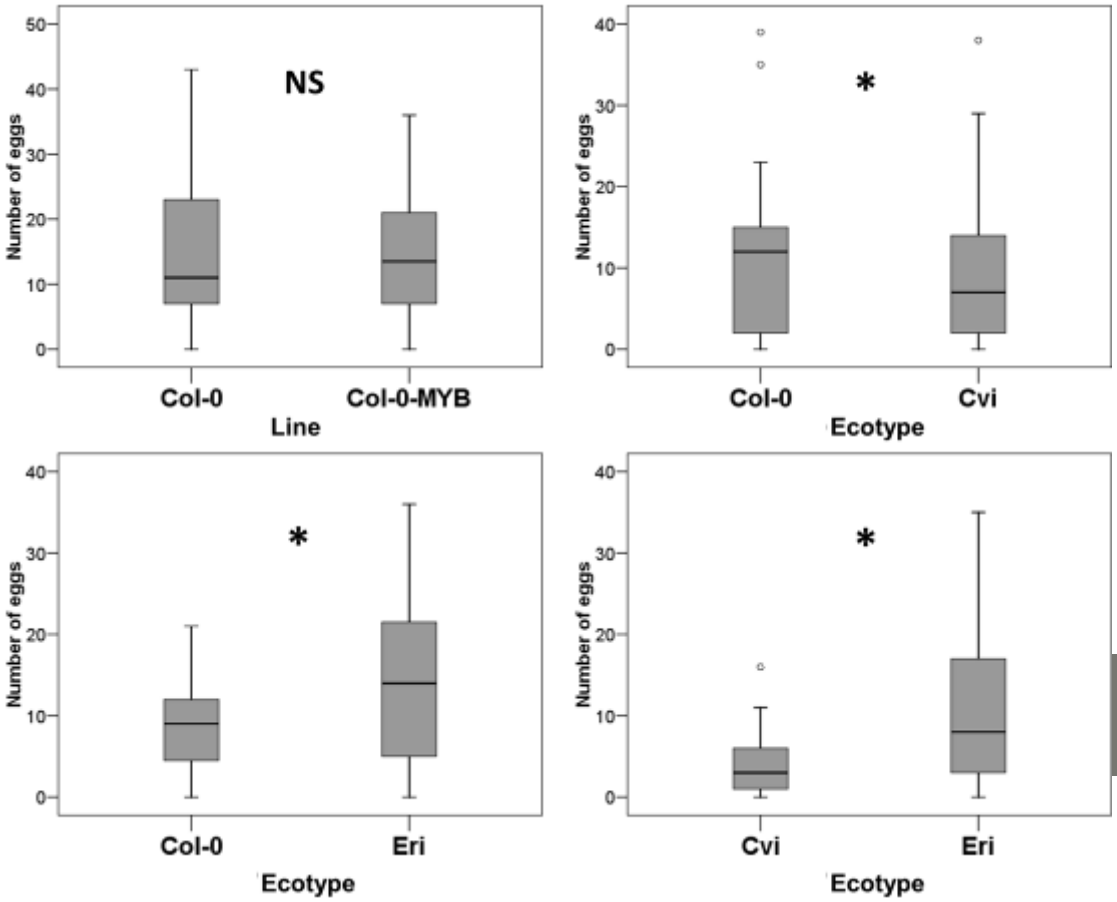


Fig. 6.4 *Oviposition preference of the aphid predator Episyrphus balteatus in a two-choice assay with aphid-infested plants of three Arabidopsis thaliana ecotypes (Col-0, Cvi and Eri) and one transformed line (Col-0-MYB28). The boxes span the first to third quartile range with the line across the box indicating the median. The whiskers represent the range. Open circles represent outliers. An asterisk indicates a significant difference (P<0.05) between the number of eggs deposited on each ecotype/line as analysed by the Wilcoxon's matched-pairs signed-rank test; NS = not significant*

every combination; Fig. 6.5). There was no effect of previous oviposition experience on the preference of the wasps (logistic regression, $P > 0.05$ for every combination).

6.4 Discussion

6.4.1 Aphid performance and GLS sequestration

The performance of *B. brassicae* was best on the *A. thaliana* ecotype with the

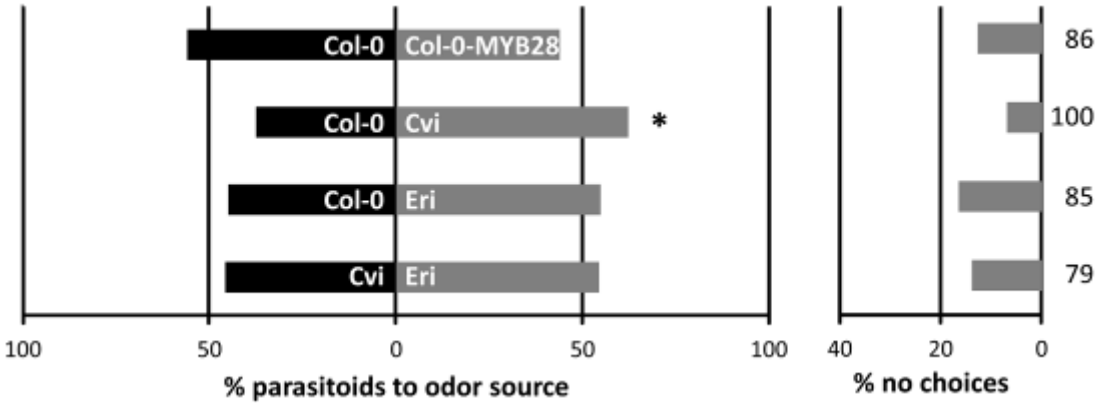


Fig. 6.5 *Responses of Diaeretiella rapae females to volatile blends emitted by aphid-infested Arabidopsis thaliana ecotypes/lines in a Y-tube olfactometer. Ecotypes investigated are: Col-0, Cvi and Eri; the transformed line is Col-0-MYB28. Each bar represents the percentage of females that made a choice for the indicated odour sources. The percentage of no choice in each experiment and the total number of tested females are indicated on the right. An asterisk indicates a significant preference for one of the two ecotypes/lines in a combination, as analysed by Chi-square tests.*

highest concentrations of aliphatic GLS in the phloem. Furthermore, we found a positive correlation between aliphatic GLS and aphid performance in the multivariate regression analysis. Due to the intercellular path taken by the aphid stylet to the phloem (Tjallingii and Hogen Esch 1993), aphids can ingest aliphatic GLS from the phloem without bringing these compounds into contact with plant myrosinases that are stored in cells adjacent to the phloem (Andreasson et al. 2001). Thus, aphids can prevent the formation of toxic hydrolytic products of aliphatic GLS (de Vos et al. 2007; Kim and Jander 2007). Together with the observation that *B. brassicae* uses GLS as feeding stimulants (Gabrys and Tjallingii 2002), our finding of a positive correlation between aliphatic GLS and aphid performance can therefore be expected. In contrast to aliphatic GLS, indole GLS have been shown to be hydrolysed in aphids into toxic products independently of myrosinase activity (Kim and Jander 2007; Kim et al. 2008), and negative correlations between the concentrations of indole GLS and performance of *B. brassicae* and other aphid species have been reported (Cole 1997; Mewis et al. 2005; Kim and Jander 2007; Kim et al. 2008). Our observation of a slight, but significant, positive correlation between aphid performance and total indole GLS

concentrations are in disagreement with these latter studies. A possible explanation for this discrepancy is that specific indole GLS may affect aphid performance more strongly than others. The difference in the abundance of specific indole GLS between our study and studies from the literature might have caused the difference in effects on aphid performance. In agreement with our finding, we also found positive correlations between indole GLS and *B. brassicae* performance in two other studies using *B. oleracea* plants (Kabouw et al. 2011; Kos et al. 2011a).

The *A. thaliana* ecotypes did not differ significantly in the concentrations of carbohydrates and amino acids in the phloem, and we did not observe a consistent correlation between aphid performance and concentrations of individual or total carbohydrates and amino acids in the regression analysis. Aphids did not seem to be affected by trichomes, as their performance was best on the *A. thaliana* ecotype with the highest trichome density. It should be noted that we measured trichome density of uninfested plants. It has been demonstrated that feeding by leaf chewers can increase trichome density in *A. thaliana* (Traw and Dawson 2002), but whether this is also true for aphids is, to our knowledge, unknown.

Brevicoryne brassicae sequestered GLS from the phloem, and total aliphatic GLS concentrations in the phloem were significantly positively correlated with their concentrations in the aphids. In contrast, whereas in the phloem 4-methoxyglucobrassicin was the most abundant indole GLS, aphids sequestered this compound to low concentrations compared to its precursor, glucobrassicin. This is in line with what we reported before in aphids feeding on *B. oleracea* plants (Kos et al. 2011b). Although the concentrations of most aliphatic GLS in the phloem were positively correlated with their concentrations in the aphids, selective sequestration was also observed for aliphatic GLS. For example, whereas gluconapin was the least abundant GLS in the phloem of Cvi plants, this compound dominated in the aphids feeding on these plants. These findings suggests that *B. brassicae* selectively sequestered GLS from the phloem, a phenomenon we reported before (Kos et al. 2011b). The mechanism underlying the selective sequestration in *B. brassicae* could be that transporters for GLS in the aphid gut wall may be rather specific. However, not much is known about the mechanism underlying GLS sequestration (Opitz et al. 2010). Aphids contained mainly aliphatic GLS, and because the hydrolysis into toxic products requires myrosinase activity circumvented by aphids (de Vos et al. 2007; Kim and Jander 2007), aphid

performance itself is most likely little affected by the high sequestration of aliphatic GLS. Aliphatic GLS are degraded more by purified aphid myrosinase, whereas the lowest activities of the aphid myrosinase are observed with indole GLS (Francis et al. 2002). We note that not all GLS detected in the aphids were detected in the phloem, probably because the concentrations of some GLS in the phloem were below the detection limit of the HPLC. However, we cannot rule out that the aphids converted certain GLS compounds from the phloem into other compounds that were subsequently stored in their body.

As expected, over-expressing Col-0-MYB28 plants produced higher foliar concentrations of aliphatic GLS and similar concentrations of indole GLS compared to the wild-type plants. Unexpectedly, we observed that aliphatic GLS concentrations in the phloem were lower in Col-0-MYB28 plants than in Col-0 plants. Probably, GLS biosynthesis in Col-0-MYB28 plants occurred mainly in the mesophyll, and phloem loading of GLS was limited. In support of our hypothesis that *B. brassicae* is not negatively affected by aliphatic GLS in the phloem, its performance was similar on Col-0 and Col-0-MYB28 plants, whereas the aliphatic GLS concentrations in the phloem differed between these two lines.

6.4.2 Predator performance

Performance of the generalist aphid predator *E. balteatus* was lowest in terms of development time when fed *B. brassicae* aphids that contained the highest aliphatic GLS concentrations (*i.e.* aphids reared on Cvi). It is likely that this led to highest concentrations of GLS hydrolysis products after breakdown by the aphid myrosinase, because purified aphid myrosinase quickly degrades aliphatic GLS (Francis et al. 2002), although we did not quantify GLS and their hydrolytic products separately. Our results are in agreement with other studies that reported negative effects of GLS sequestration by *B. brassicae* on the performance of *E. balteatus* (Vanhaelen et al. 2002; Kos et al. 2011b) and other aphid predators (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008; Chaplin-Kramer et al. 2011; Kos et al. 2011b). Hoverfly performance did not differ between Col-0 and Col-0-MYB28 plants. This was expected because aphid GLS concentrations did not differ between these plant lines.

Predator performance in terms of development time was better when their prey had been feeding on ecotype Eri than on Col-0. However, Eri-fed aphids had higher total GLS concentrations in their phloem than Col-0-fed aphids, although the difference was not statistically different. This points to the

importance of qualitative effects of GLS profiles on hoverfly performance. It has been reported before that differences in aphid GLS profiles can affect the performance of *E. balteatus* (Kos et al. 2011b). However, we cannot rule out effects of other plant or aphid traits on predator performance. It has been shown before that trichomes can negatively affect the performance of hoverfly larvae due to entrapment by glandular trichomes, reduced mobility or falling off the plant (Verheggen et al. 2009). Although we do not know whether this is also true for non-glandular trichomes on *A. thaliana*, the lower trichome density of Eri plants might have contributed to the better performance of hoverfly larvae on this ecotype.

6.4.3 Parasitoid performance

Parasitoid performance in terms of adult weight was best when developing in the largest aphids, containing the highest GLS concentrations. The positive correlation between host size and parasitoid size is in agreement with other studies (Harvey 2005; Bukovinszky et al. 2008). Our results suggest that the performance of *D. rapae* is not negatively affected by GLS concentrations in the host, supporting the findings of Le Guigo et al. (2011). Although *D. rapae* parasitizes several aphid species, it is the main parasitoid of *B. brassicae* (Bukovinszky et al. 2008), and may be relatively tolerant to GLS. We do, however, not know how *D. rapae* copes with GLS in its host. In fact, there is not much known about detoxification of plant secondary metabolites by parasitoids in general (Ode 2006; Gols and Harvey 2009). Negative effects of breakdown products of GLS on *D. rapae* might be prevented by the feeding strategy of the parasitoid larvae. *Brevicoryne brassicae* stores the GLS in the haemolymph, but the aphid's myrosinases are stored in the non-flight muscles (Jones et al. 2001; Bridges et al. 2002; Francis et al. 2002). Tissue-feeding endoparasitoids, such as *D. rapae*, consume host haemolymph during most of their larval development and only consume other host tissues shortly before egression (Godfray 1994; Harvey et al. 2000), thereby possibly preventing the breakdown of GLS into toxic products during the major part of their development. *Diaeretiella rapae* performance did not differ between Col-0 and Col-0-MYB28 plants, which was expected as both aphid size and aphid GLS concentrations did not differ between these lines.

6.4.4 Natural enemy preference and volatile emission

Aphid-infested plants of the three *A. thaliana* ecotypes differed in their volatile

profiles. Both the predator (*E. balteatus*) and the parasitoid wasp (*D. rapae)* preferred the ecotype on which their offspring performed best. This demonstrates that preference and performance of these natural enemies are positively correlated, in agreement with other studies (Soler et al. 2007; Gols et al. 2009). The parasitoid preferred volatile cues from the ecotype with the highest emission of volatile GLS hydrolysis products (Cvi) over cues from the ecotype with the lowest emission of volatile GLS hydrolysis products (Col-0). This was expected, as *D. rapae* is known to be attracted to host plants emitting volatile breakdown products of GLS (Read et al. 1970; Bradburne and Mithen 2000; Blande et al. 2007). In contrast, the predator laid fewest eggs on Cvi, suggesting that volatile breakdown products of GLS were repellent for the predators. *Episyrphus balteatus* had access to the aphid-infested plants in the bioassays. We do not know if other plant characteristics or aphid cues also played a role in the selection of an oviposition site by *E. balteatus*. In particular, the preferred ecotype had the lowest trichome density. It has been shown before that adult hoverflies have problems with landing on plants with high trichome densities (Verheggen et al. 2009). Neither the parasitoid wasp nor the predator differentiated between cues from Col-0 and Col-0-MYB28 plants. The relatively small difference in volatile profiles between these lines might not allow olfactory discrimination. In a PLS-DA including the volatile profiles of the three ecotypes and Col-0-MYB28, the profile of Col-0-MYB28 was very similar to the profile of Col-0, as compared to the other ecotypes (**Chapter 10**).

6.4.5 Conclusion

In conclusion, the four main findings of our study are: 1) The performance of the specialist cabbage aphid *B. brassicae* was positively correlated with the concentrations of both aliphatic and indole GLS in the phloem of *A. thaliana* plants; 2) *Brevicoryne brassicae* selectively sequestered GLS from the phloem; 3) The performance of the aphid predator *E. balteatus* was negatively correlated with aphid GLS concentrations. The performance of the aphid parasitoid *D. rapae* was positively correlated with aphid GLS concentrations, probably because the aphids with the highest GLS concentrations had a higher body weight; 4) Both natural enemies preferred the *A. thaliana* ecotype on which their offspring performed best, indicating a positive performance-preference correlation. The parasitoid wasp preferred the *A. thaliana* ecotype with the highest emission of volatile breakdown products of GLS, while the

predator selected against this ecotype. Our study shows that there are differential herbivore-mediated effects of GLS on a predator and a parasitoid of a specialist aphid that selectively sequesters GLS from its host plant.

6.5 Acknowledgements

We thank Ana Pineda, Meindert van der Wielen and Qianjue Wang for practical assistance; Prof. Flügge (University of Cologne, Germany) for providing the *MYB28* cDNA and Dr. Beekwilder for communications with Prof. Flügge; Koppert Biological Systems for providing *E. balteatus* and Unifarm for rearing the Brussels sprouts plants. This work was supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (number 838.06.010).

7

Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an associated parasitoid

Martine Kos, Benyamin Houshyani, Rafal Wietsma, Patrick Kabouw, Louise E.M. Vet, Joop J.A. van Loon and Marcel Dicke

Published in a slightly different form in *Phytochemistry* (2012), doi: 10.1016/j.phytochem.2012.01.005

Abstract

Glucosinolates (GLS) are secondary plant metabolites that as a result of tissue damage caused by herbivore feeding are hydrolysed into toxic compounds that negatively affect generalist herbivores, whereas specialist herbivores have evolved specific adaptations to detoxify GLS or inhibit the formation of toxic hydrolytic products. Although rarely studied, GLS and their breakdown products may also affect parasitoids. The objectives were to test the effects of GLS in a multitrophic system consisting of the generalist herbivore *Spodoptera exigua*, the specialist herbivore *Pieris rapae*, and the endoparasitoid *Hyposoter ebeninus*. Several ecotypes of *Arabidopsis thaliana* that differ in their GLS composition and concentrations and one transformed line that constitutively produces higher concentrations of aliphatic GLS were used, the latter allowing a direct assessment of the effects of aliphatic GLS on herbivore and parasitoid performance.

Feeding by the generalist *S. exigua* and the specialist *P. rapae* induced both higher aliphatic and indole GLS concentrations in the *A. thaliana* ecotypes, although induction was stronger for indole than aliphatic GLS. For both herbivores a negative correlation between performance and aliphatic GLS concentrations was observed. This suggests that *P. rapae*, despite that it contains a nitrile-specifier protein (NSP) in the gut that diverts GLS degradation from toxic isothiocyanates to less toxic nitriles, cannot completely inhibit the formation of toxic GLS hydrolytic products. Surprisingly, performance of the parasitoid was positively correlated with higher concentrations of aliphatic GLS in the plant, possibly caused by negative effects on host immune responses. Our study indicates that GLS can not only confer resistance against herbivores directly, but also indirectly by increasing the performance of the parasitoids of these herbivores.

7.1 Introduction

Plants have evolved a wide array of resistance traits that prevent or reduce insect herbivory. These traits can affect the performance or behaviour of herbivores directly by chemical means, such as the production of toxins, repellents and digestibility reducers, or by physical means, such as the production of trichomes and epicuticular waxes (Karban and Baldwin 1997; Schoonhoven et al. 2005). Plants can also affect herbivores indirectly, by promoting the effectiveness of natural enemies that feed on these herbivores. For example, plants emit herbivore-induced volatiles that attract natural enemies of herbivores to the plant (Vet and Dicke 1992; Schoonhoven et al. 2005; Dicke and Baldwin 2010; Hare 2011; Kessler and Heil 2011). Secondary plant metabolites that confer direct resistance to herbivores can also negatively influence the performance of the natural enemies of these herbivores, either directly due to toxicity of the compounds, or indirectly due to reduced growth and development of their host or prey (Harvey 2005; Ode 2006; Gols and Harvey 2009).

Among the best studied secondary plant metabolites are glucosinolates (GLS) that are characteristic for the plant family Brassicaceae. Upon damage, the GLS that are stored in the vacuoles become exposed to the enzyme myrosinase, which is stored separately in special cells. As a result of the myrosinase activity, GLS are hydrolysed into several toxic compounds such as (iso)thiocyanates and nitriles. These breakdown products negatively affect a wide variety of generalist herbivores (Bones and Rossiter 2006; Halkier and Gershenzon 2006; Hopkins et al. 2009). Specialist herbivores of Brassicaceae, however, have evolved specific adaptations to detoxify GLS or inhibit the formation of toxic (iso)thiocyanates (Ratzka et al. 2002; Wittstock et al. 2004), sequester GLS (Francis et al. 2001b; Kazana et al. 2007; Müller 2009; Kos et al. 2011b; 2012), or use GLS and their hydrolysis products as oviposition or feeding stimulants (van Loon et al. 1992; Gabrys and Tjallingii 2002; Miles et al. 2005).

GLS and their breakdown products may not only affect herbivores, but also natural enemies, such as predators and parasitoids, that feed on GLS-containing herbivores. Most studies on the effects of GLS on natural enemies involved predators, and these studies reported negative effects of GLS and their breakdown products on the performance of predators (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008; Chaplin-Kramer et al. 2011; Kos et al. 2011b; 2012). Parasitoids, however, have been studied less frequently, and

little is known about the effects of GLS and their breakdown products on the performance of parasitoids (Gols and Harvey 2009).

The objectives of this study were to test the effects of GLS in a multitrophic system consisting of the brassicaceous *Arabidopsis thaliana* (L.) Heynh., the generalist herbivore *Spodoptera exigua* Hübner (Beet Armyworm: Lepidoptera, Noctuidae), the specialist herbivore *Pieris rapae* L. (Small Cabbage White butterfly; Lepidoptera: Pieridae), and the solitary koinobiont endoparasitoid *Hyposoter ebeninus* Gravenhorst (Hymenoptera: Ichneumonidae). *Spodoptera exigua* feeds on many plant species, among which brassicaceous plants, and is negatively affected by GLS and their breakdown products (Gigolashvili et al. 2007; Arany et al. 2008; Müller et al. 2010). The brassicaceous specialist *P. rapae* possesses a nitrile-specifier protein (NSP) that diverts GLS degradation from toxic isothiocyanates to less toxic nitriles that are excreted with the faeces (Wittstock et al. 2004). *Pieris rapae* adults use GLS as oviposition stimulants, and their larvae use these compounds as feeding stimulants (Miles et al. 2005; Müller et al. 2010). The endoparasitoid *H. ebeninus* parasitizes, among others, the larvae of *P. rapae*. Adult females lay a single egg into a host larva. The hatched parasitoid larva first feeds on haemolymph and fat body of the host, whereas later in development it consumes all host tissues (except the head capsule and the outer layer of the cuticle), which leads to the death of the host (Harvey et al. 2010; J. Harvey, personal communication). The biology of *H. ebeninus* has only recently been studied in detail (Harvey et al. 2010) and, as far as we know, herbivore-mediated effects of GLS on *H. ebeninus* have never been tested.

To provide the herbivores with plants that differ in GLS content, several ecotypes of *A. thaliana* that differ in their total GLS concentrations, as well as in their GLS profiles (the qualitative and quantitative composition of the mix of GLS) were used (Houshyani et al. 2012). Furthermore, a genetically transformed line was developed that produces higher concentrations of aliphatic GLS compared to the wild-type plant. This allowed for making a direct assessment of the effects of aliphatic GLS concentrations on herbivore and parasitoid performance. It was expected that the performance of both herbivores would be negatively affected by higher levels of GLS in the host plant, but more so for the generalist *S. exigua* than for the specialist *P. rapae*. Furthermore, it was expected that GLS, through direct negative effects as well as through negative effects on the performance of *P. rapae*, would also

negatively affect performance of *H. ebeninus*. The cascading effects of GLS in this multitrophic system are discussed.

7.2 Materials and methods

7.2.1 Plant material and growth conditions

Three *Arabidopsis thaliana* ecotypes were selected that differ in their GLS concentrations and profiles (Houshyani et al. 2012): Columbia (Col)-0 (provided by Dr. P. Reymond, Lausanne, Switzerland), Cape Verde Island (Cvi; obtained from the European Arabidopsis Stock Centre, http:// nasc.nott.ac.uk/, Cvi = N8580) and Eringsboda (Eri; collected in Sweden by members of the Laboratory of Genetics, Wageningen University; Eri-1 = CS22548).

To produce plants with higher foliar levels of aliphatic GLS we over-expressed the transcription factor HAG1/MYB28 in *A. thaliana* ecotype Col-0 (Houshyani et al., in prep.; see also Method S6.1 in the Supporting Information of **Chapter 6**. This transcription factor represents a key component in the regulation of aliphatic GLS biosynthesis in *A. thaliana* (Gigolashvili et al. 2007). T2 generation seeds of one successfully transformed line (hereafter named Col-0-MYB28) were used in the experiments.

Arabidopsis thaliana seeds were surface-sterilized overnight by vapour phase sterilization and inoculated on a growth medium (purified agar 0.8% + 2.2 g L^{-1} 0.5 MS + vitamins; pH 6; containing 30 µg ml^{-1} kanamycin to select transformed seedlings). After four days of stratification at 4 °C, plates were transferred to a growth chamber at 21 ± 2 °C, 50-70% relative humidity (RH) and a 8:16 light:dark (L:D) photoregime with a light intensity of 200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diameter) containing autoclaved soil (80 °C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times per week and entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were added weekly to the soil to control infestation by larvae of sciarid flies. Plants were used in the experiments when they were seven weeks old and remained in the vegetative state during the experiments.

7.2.2 Insect rearing

Spodoptera exigua was reared on artificial diet (Table S7.1 in the Supporting

Information) in a climatized room at 27 ± 2 °C, 50% RH and a 16:8 h L:D photoregime. Adults were provided with water-saturated cotton wool. *Pieris rapae* was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) in a climatized room at 22 ± 2 °C, 40-50% RH and a 16:8 h L:D photoregime. Adults were provided with a sugar water solution in small plastic tubes. Female *S. exigua* and *P. rapae* were allowed to oviposit on a piece of filter paper (*S. exigua*) or a Brussels sprouts plant (*P. rapae*) for 24 h. Neonate larvae were used in the experiments.

Hyposoter ebeninus was reared in *P. rapae* larvae. A leaf of a Brussels sprouts plant infested with second instar *P. rapae* larvae was exposed to female parasitoids for approximately one hour. Afterwards, the parasitized larvae were transferred to a gauze cage (30 x 40 x 60 cm) containing Brussels sprouts plants in a greenhouse compartment at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime. Parasitoid cocoons were collected from the plants and the eclosed adult wasps were provided with water and honey. Because the survival of *S. exigua* was very low (see *Results*), it was not possible to include a parasitoid of *S. exigua* in our study.

7.2.3 GLS analysis

To correlate herbivore performance with GLS profiles of the plants, GLS were extracted from 10 uninfested, 10 *S. exigua*-infested and 10 *P. rapae*-infested plants of each ecotype/line. Infested plants had been inoculated by one neonate larva. After five days, the larvae were removed from the plants and the remaining leaf material was harvested for GLS extraction. GLS could not be extracted from the same plants on which herbivore performance was tested, because there was not enough leaf material left after feeding by the herbivores during their entire larval development. Leaf samples were frozen at -80 °C immediately after collection, freeze-dried, weighed into a micro-centrifuge tube and ground to a fine powder. GLS were extracted and purified by using the methods of van Dam et al. (2004) and Kabouw et al. (2010a) and foliar GLS content was assessed using high-performance liquid chromatography (HPLC). GLS detection was performed with a photodiode array detector set at 229 nm as the integration wavelength. Different concentrations of sinigrin (Acros, Morris Plains, New Jersey, USA) were used as an external standard. The correction factors at 229 nm from Buchner (1987) and the European Community (1990) were used to calculate the concentrations of the GLS. DesulfoGLS peaks were identified by comparison

of HPLC retention times and ultraviolet spectra with standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology, Jena, Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels, Belgium, code BCR-367 R).

7.2.4 Insect performance

The performance of *S. exigua*, *P. rapae* and *H. ebeninus* was tested in no-choice situations in cylindrical plastic containers (height 13 cm; diameter 11 cm) with a gauze lid, each containing one *A. thaliana* plant. The experiments were performed in a climate chamber at 21 ± 2 °C, 50-70% RH and a 16:8 L:D photoregime with a light intensity of 200 µmol m^{-2} s^{-1} PPFD inside the container. Plants were watered once per week.

7.2.4.1 Spodoptera exigua and Pieris rapae performance

Forty plants of each ecotype/line were infested with one neonate *S. exigua* larva and 25 plants with one neonate *P. rapae* larva. A larger number of replicates was used for *S. exigua* because a higher mortality of this generalist herbivore was expected. Larvae were allowed to feed on the plant until pupation. Additional plants were added if the larvae had consumed the first plant. Pupae were left in the container until adult eclosion, which was checked once a day, and survival, neonate-to-adult development time, sex and adult dry weight were measured. Adult dry weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany) by weighing freshly eclosed adults that had been dried to constant weight at 80 ˚C for 3 days.

7.2.4.2 Hyposoter ebeninus performance

Twenty mated female *H. ebeninus* adults of five-to-seven days old were collected from the stock rearing. Second instar (three day old) *P. rapae* larvae that were reared on one of each of the *A. thaliana* ecotypes/lines were exposed individually to a female parasitoid on a feeding-damaged leaf until parasitisation was observed (*i.e.* when the female inserted her ovipositor in the larva). Two parasitized larvae were transferred to one *A. thaliana* plant of the same ecotype/line as the one on which these larvae had been reared. Two larvae per plant instead of one were used because it was expected that parasitisation of the larvae would increase their mortality. In total 40 plants per *A. thaliana* ecotype/line were tested. Parasitoid cocoons were collected from the plants and upon adult parasitoid eclosion, parasitoid sex was determined,

and egg-to-adult development time and adult dry weight were measured (similar as described above). Survival of the parasitized *P. rapae* larvae, either until host pupation or until *H. ebeninus* eclosion, was calculated by adding the number of hosts that pupated to the number of hosts that developed into a *H. ebeninus* adult and dividing this number by the total number of larvae that were tested. The percentage of successful parasitism of *P. rapae* larvae by *H. ebeninus* was calculated by dividing the number of *H. ebeninus* adults by the total number of *P. rapae* larvae that survived (either until host pupation or until *H. ebeninus* eclosion).

7.2.5 Statistical analysis

Analyses were performed in SPSS for Windows (15th edition, Chicago, Illinois, USA), unless indicated otherwise. Following each ANOVA performed in this study, post-hoc Tukey-tests were used for pair-wise comparisons.

GLS concentrations were log-transformed to obtain normality. Differences in aliphatic and indole GLS concentrations of leaves a) among the ecotypes and b) between Col-0 and Col-0-MYB28 were analysed by two-way ANOVA for the factors plant ecotype/line, herbivore treatment (uninfested, *P. rapae*-infested and *S. exigua*-infested) and the interactions. Because the only aromatic GLS (gluconasturtiin) detected was present in only trace amounts, this compound was excluded from statistical analysis.

To test if there were differences in GLS profiles of leaves a) among the nine *A. thaliana* ecotype x herbivore treatment combinations and b) among the six *A. thaliana* line (Col-0 and Col-0-MYB28) x herbivore treatment combinations, the multivariate analysis Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12th edition, Umetrics, Umeå, Sweden) (Eriksson et al. 2006) was used. To pre-process data, GLS concentrations were mean-centred and scaled to unit variance.

Differences in development time and adult dry weight of the herbivores and parasitoids a) among the ecotypes and b) between Col-0 and Col-0-MYB28 were analysed by two-way ANOVA on the factors plant ecotype/line, sex, and the interactions. The development time of *P. rapae* was log-transformed to obtain normality. Differences in the survival and the percentage of successful parasitism a) among the ecotypes and b) between Col-0 and Col-0-MYB28 were analysed by logistic regression in GenStat (13h edition, VSN International, UK), followed by calculating t-probabilities to test pair-wise differences between means.

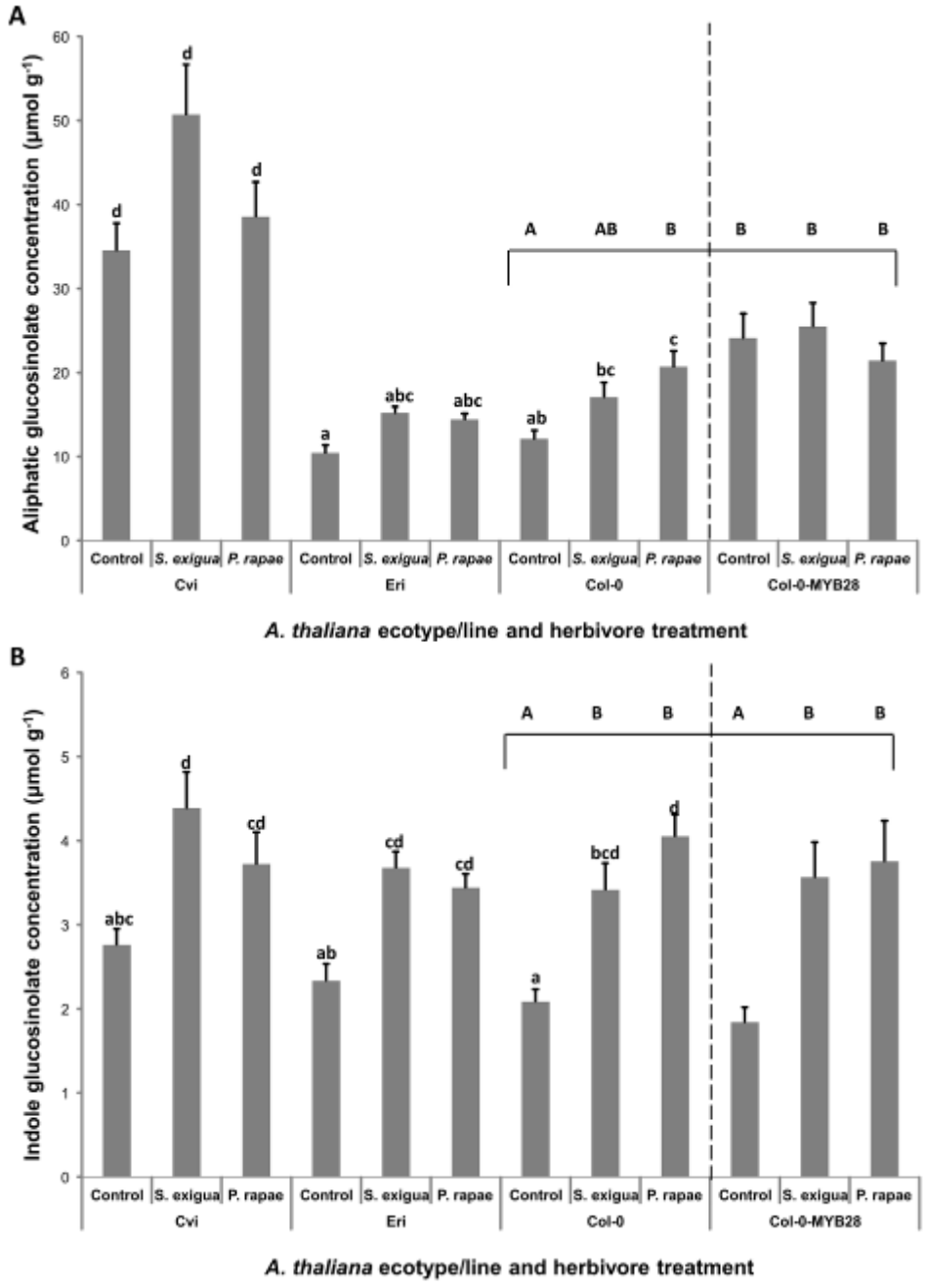


Fig. 7.1. *Aliphatic (A) and indole (B) glucosinolate (GLS) concentration (in µmol g^{-1} dry weight; mean + SE) of plants of three Arabidopsis thaliana ecotypes (Cvi, Eri and Col-0) and one transformed line that produces higher levels of aliphatic GLS (Col-0-MYB28). Plants were either uninfested (control), infested for five days by one neonate Spodoptera exigua larva or infested for five days by one neonate Pieris rapae larva. GLS were divided into indole and aliphatic GLS based on their biosynthetic origin. n = 10 for each bar. Different small case letters indicate differences between the nine ecotype x herbivore treatment combinations at the level of P < 0.05 (post-hoc Tukey tests). Different capital letters are used to compare Col-0 and Col-0-MYB28 plants and indicate differences between the six line x herbivore treatment combinations at the level of P < 0.05 (post-hoc Tukey tests).*

7.3 Results

7.3.1 GLS analysis

7.3.1.1 Aliphatic and indole GLS

Ecotype effect: The three *A. thaliana* ecotypes differed in the concentrations of aliphatic GLS, but not in indole GLS, as averaged for uninfested and herbivore -infested plants (ANOVA, aliphatic: $F_{2,81}$ = 123.81, P < 0.001; indole: $F_{2,81}$ = 2.33, P = 0.104). Cvi plants contained higher concentrations of aliphatic GLS than Eri and Col-0 plants (Fig. 7.1). Herbivore feeding increased concentrations of aliphatic and indole GLS, as averaged over the three ecotypes (ANOVA, aliphatic: $F_{2,81}$ = 14.07, P < 0.001; indole: $F_{2,81}$ = 30.48, P < 0.001). There were no differences in induction of aliphatic or indole GLS between the two herbivore species (post-hoc Tukey tests on the effect of herbivore treatment, P > 0.05 for both comparisons). There was no interaction between ecotype and herbivore treatment for aliphatic and indole GLS (ANOVA, P > 0.05 for both analyses). The pairwise differences in GLS concentrations among the nine ecotype x herbivore treatment combinations can be seen in Fig. 7.1. Based on the number of statistically significant pairwise differences in GLS concentrations between uninfested and infested plants, the induction effects of herbivory were most apparent for indole GLS (Fig. 7.1).

Over-expression effect: As averaged over the three herbivore treatments, Col-0-MYB28 plants contained higher aliphatic GLS concentrations than Col-0 plants (ANOVA, $F_{2,54}$ = 19.18, P < 0.001). However, there was an interaction between the effect of the *A. thaliana* line (Col-0 or Col -0-MYB28) and the herbivore treatment (ANOVA, $F_{2,54}$ = 5.38, P = 0.007), because herbivore feeding did not induce aliphatic GLS in Col-0-MYB28 plants, whereas it did in Col-0 plants. As a result, aliphatic GLS concentrations did not differ between herbivore-infested Col-0 and herbivore-infested Col-0-MYB28 plants (Fig. 7.1). Concentrations of indole GLS did not differ between Col-0 and Col-0-MYB28 plants, but were induced significantly by herbivore feeding in both Col-0 and Col-0-MYB28 plants (ANOVA, herbivore treatment: $F_{2,54}$ = 22.86, P < 0.001; *A. thaliana* line and interaction: P > 0.05; Fig. 7.1).

7.3.1.2 GLS profiles

Ecotype effect: GLS profiles differed among the nine plant ecotype x herbivore treatment combinations (3 PLS-DA principal components, R_2X_{cum} = 0.920, R_2Y_{cum} = 0.316, Q_{2cum} = 0.292). The PLS-DA model showed that the largest

difference in GLS profiles was due to the ecotype effect, and that the largest difference among the ecotypes was between Cvi and the other two ecotypes (Fig. 7.2). Differences among the ecotypes were both qualitative (different compounds) and quantitative (different concentrations of the compounds). Feeding by *S. exigua* or *P. rapae* only slightly, and only quantitatively, changed the GLS profile of the plant compared to the profile of the uninfested control plants. There were no clear differences in GLS profiles between plants infested by *S. exigua* or *P. rapae* (Fig. 7.2).

Over-expression effect: Also when comparing Col-0 and Col-0-MYB28, GLS profiles differed among the different plant line x herbivore treatment combinations (3 PLS-DA principal components, R_2X_{cum} = 0.772, R_2Y_{cum} = 0.375, Q_{2cum} = 0.332). There were only quantitative differences in GLS profiles between the two lines, as both lines produced the same GLS compounds, but in different concentrations. Again, herbivore feeding only quantitatively changed the GLS profile of the plants, and there were no clear differences in GLS profiles between plants infested by *S. exigua* or *P. rapae* (Fig. 7.2).

7.3.2 *Spodoptera exigua* performance

Ecotype effect: Survival of *S. exigua* until the adult stage was on average 33% (25 adults out of 75 larvae), and did not differ among the ecotypes (logistic regression, $P > 0.05$, Table 7.1). *Spodoptera exigua* developed fastest into the adult stage on Eri plants, at intermediate rate on Col-0 plants, and slowest on Cvi plants (ANOVA, $F_{2,19} = 53.80$, $P < 0.001$; Fig. 7.3). Adult body weight of *S. exigua* was highest on Eri plants, intermediate on Col-0 plants, and lowest on Cvi plants (ANOVA, $F_{2,19} = 27.27$, $P < 0.001$; Fig. 7.3). There was no effect of sex or the interaction between ecotype and sex on development time and adult dry weight (ANOVA, $P > 0.05$ for every analysis).

Over-expression effect: survival of *S. exigua* was twice lower on Col-0 -MYB28 plants than on Col-0 plants, but the difference was not statistically different (logistic regression, $P > 0.05$; Table 7.1). *Spodoptera exigua* neonate-to-adult development time and adult dry weight did not differ between Col-0 and Col-0-MYB28 plants (ANOVA, $P > 0.05$ for both comparisons; Fig. 7.3).

7.3.3 *Pieris rapae* performance

Ecotype effect: Survival of *P. rapae* until the adult stage was on average 77% and did not differ among the ecotypes (logistic regression, $P > 0.05$; Table

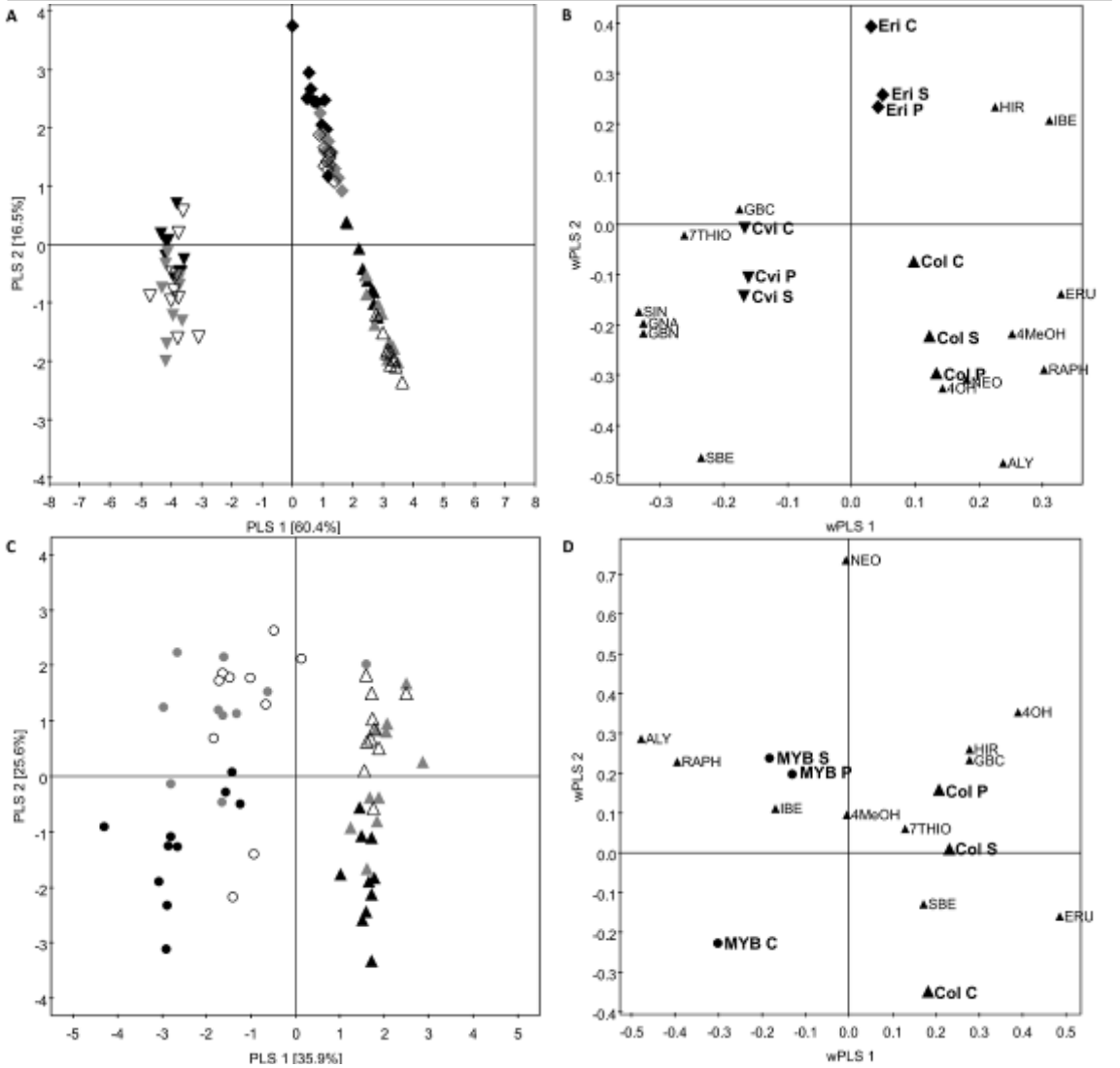


Fig. 7.2. *PLS-DA score and loading plots of the first two components showing the glucosinolate (GLS) profiles of three Arabidopsis thaliana ecotypes (**A** and **B**) and one A. thaliana ecotype and its transformed line that produces higher levels of aliphatic GLS (**C** and **D**). Plant ecotypes are Cvi (inverted triangles), Eri (diamonds) and Col-0 (triangles); transformed line is Col-0-MYB28 (circles). Herbivore treatments are: uninfested control (black labels in score plot, C in loading plot), infested for five days by one neonate Spodoptera exigua larvae (grey labels, S) or infested for five days by one neonate Pieris rapae larva (white labels, P). The score plots (**A** and **C**) show the distinction in GLS profiles of the different ecotype/line x herbivore treatment combinations. In brackets the percentage of variation explained is indicated for each axis. The loading plots (**B** and **D**) show the contribution of each of the GLS compounds to the discrimination among the different ecotype/line x herbivore treatment combinations. Aliphatic GLS: ALY = glucoalyssin, GBN = glucobrassicanapin, GNA = gluconapin, HIR = glucohirsutin, IBE = glucoiberin, ERU = glucoerucin, RAPH = glucoraphanin, SBE = glucosiberin, SIN = sinigrin. 7THIO = 7-methylthioheptylGLS. Indole GLS: GBC = glucobrassicin, NEO = neo-glucobrassicin, 4MeOH = 4-methoxyglucobrassicin, 4OH = 4-hydroxyglucobrassicin. See Table S6.2 in the Supporting Information of **Chapter 6** for the scientific names of all GLS compounds.*

Table 7.1. Performance parameters of *Spodoptera exigua*, *Pieris rapae* and *Hyposoter ebeninus* reared on three *Arabidopsis thaliana* ecotypes and one transformed line that produces higher levels of aliphatic glucosinolates

		***A. thaliana* ecotype**[a]			**Transformed line**[b]
Species	**Performance parameter**	**Cvi**	**Eri**	**Col-0**	**Col-0-MYB28**
S. exigua	Survival until adult stage (%)	28 **a**	32 **a**	40 **a**	20 **ns**
	Number of individuals (*n*) surviving until adult stage	7	8	10	5
P. rapae	Survival until adult stage (%)	68 **a**	80 **a**	84 **a**	56 *
	Number of individuals (*n*) surviving until adult stage	17	20	21	14
H. ebeninus	Survival until host pupation/*H. ebeninus* eclosion (%)	70 **a**	75 **a**	58 **a**	70 **ns**
	Successful parasitism (%)	71 **b**	48 **a**	63 **ab**	46 **ns**
	Number of individuals (*n*) surviving until adult stage	40	29	31	29 **ns**
	Egg-to-adult development time (days; mean ± SE)	17.2 ± 0.1 **a**	17.0 ± 0.1 **a**	17.4 ± 0.1 **a**	17.3 ± 0.1 **ns**
	Adult dry weight (mg; mean ± SE)	2.17 ± 0.04 **a**	2.27 ± 0.08 **a**	2.13 ± 0.07 **a**	2.06 ± 0.04 **ns**

*[a] Different letters denote differences in means among the three ecotypes as analysed by logistic regression and post-hoc T-probability tests (survival) or ANOVA (development time and dry weight); [b] * denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analysed by logistic regression and post-hoc T-probability tests (survival) or ANOVA (development time and dry weight).*

7.1). *Pieris rapae* development time was affected by plant ecotype and the interaction between ecotype and sex, but not by sex itself (ANOVA, ecotype: $F_{2,52} = 30.79$, $P < 0.001$; sex: $F_{1,52} = 1.83$, $P = 0.182$; interaction: $F_{2,52} = 3.70$, $P = 0.032$). Overall, *P. rapae* developed faster into the adult stage on Col-0 and Eri plants than on Cvi plants (Fig. 7.3). The interaction between ecotype and sex was due to a slightly faster development of females on Eri plants compared to males, whereas females on Col-0 and Cvi developed slightly slower than males, although none of the differences between males and females were significant. Adult dry weight of *P. rapae* was affected only by plant ecotype, and not by sex or the interaction between both (ANOVA, ecotype: $F_{2,52} = 11.41$, $P < 0.001$; sex and interaction $P > 0.05$). Adult dry weight of *P. rapae* was higher on Eri and Col-0 plants than on Cvi plants (Fig. 7.3).

Over-expression effect: survival of *P. rapae* was lower on Col-0-MYB28 plants than on Col-0 plants (logistic regression, *d.f.* = 1, deviance ratio = 4.05, $P = 0.044$; Table 7.1). Development time and adult dry weight differed between Col-0 and Col-0-MYB28 plants (ANOVA, development time: $F_{1,31} = 9.82$, $P = 0.004$; weight: $F_{1,31} = 7.30$, $P = 0.011$). *Pieris rapae* developed slower and into adults with a lower body weight on Col-0-MYB28 plants (Fig. 7.3).

7.3.4 *Hyposoter ebeninus* performance

Ecotype effect: Survival of the parasitized *P. rapae* larvae, either until host pupation or until *H. ebeninus* eclosion, was on average 68% and did not differ among the ecotypes (logistic regression, $P > 0.05$, Table 7.1). The percentage of successful parasitism of *P. rapae* larvae by *H. ebeninus* differed among the ecotypes (logistic regression, *d.f.* = 2, deviance ratio = 3.69, $P = 0.025$) and was higher on Cvi plants than on Eri plants (Table 7.1). Furthermore, the percentage of successful parasitism was higher on Col-0 plants than on Eri plants (Table 7.1), although this difference was only marginally significant ($P = 0.051$). Females developed slower than males (17.5 ± 0.1 days and 17.2 ± 0.1 days respectively) (ANOVA, $F_{1,93} = 6.51$, $P = 0.012$). Females had a higher body weight than males (2.67 ± 0.09 mg and 2.10 ± 0.03 mg respectively) (ANOVA, $F_{1,93} = 65.17$, $P > 0.001$). Although the development time and dry wet of the host differed among the plant ecotypes, there was no difference in the egg-to-adult development time or adult dry weight of *H. ebeninus* among the plant ecotypes (Table 7.1), nor a significant interaction between parasitoid sex and plant ecotype (ANOVA, $P > 0.05$ for every analysis).

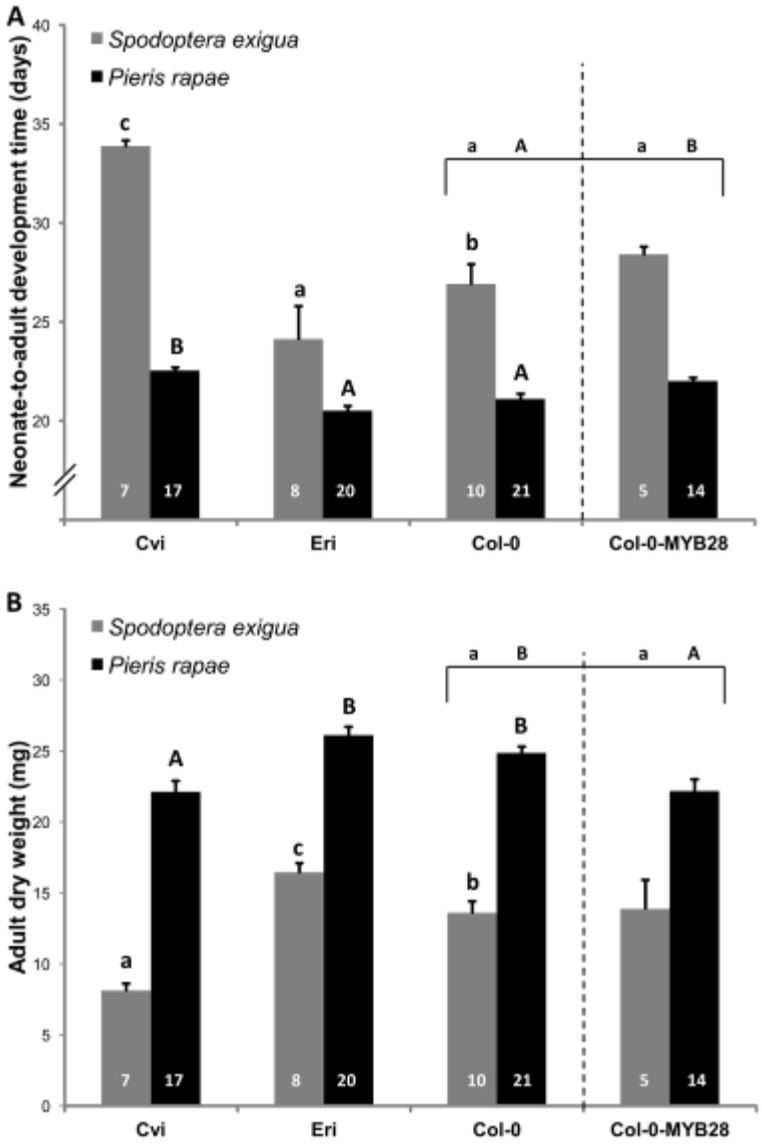


Fig. 7.3. *Neonate-to-adult development time in days (A) and adult dry weight in mg (B) (mean + SE) of Spodoptera exigua and Pieris rapae when feeding on three Arabidopsis thaliana ecotypes (Cvi, Eri and Col-0) and one transformed line that that produces higher levels of aliphatic glucosinolates (Col-0-MYB28). Within a herbivore species, different letters (small letters for S. exigua and capital letters for P. rapae) above the bars indicate significant differences among the A. thaliana ecotypes at the level of P < 0.05 (post-hoc Tukey tests). Differences in herbivore performance between Col-0 and Col-0-MYB28 plants are indicated by the letters above the horizontal line (small letters for S. exigua and capital letters for P. rapae). n tested is indicated in each bar.*

Over-expression effect: Survival of the *P. rapae* larvae until host pupation or until *H. ebeninus* eclosion and the percentage of successful parasitism did not differ between Col-0 and Col-0-MYB28 plants (logistic regression, $P > 0.05$ for both comparisons, Table 7.1). Although the development time and dry weight of the host differed between Col-0 and Col-0 -MYB28 plants, there were no differences in egg-to-adult development time and adult dry weight between parasitoids reared on both lines (ANOVA, $P > 0.05$ for both comparisons; Table 7.1).

7.4 Discussion

This study tested if effects of GLS cascaded in a multitrophic system consisting of a generalist and a specialist leaf-chewing herbivore and an associated parasitoid of the latter. To provide the herbivores with plants that differed in GLS content, three ecotypes of *A. thaliana* that differed in aliphatic GLS concentrations, but not in indole GLS concentrations, were used. The ecotypes also differed in GLS profiles, *i.e.* the qualitative and quantitative composition of the mix of GLS. Furthermore, a genetically transformed line was developed that produced higher concentrations of aliphatic GLS than the corresponding wild-type, but similar concentrations of indole GLS (Col-0-MYB28), allowing a direct assessment of the effects of aliphatic GLS on herbivore and parasitoid performance.

7.4.1 Induction of GLS by herbivory

Feeding by the generalist *S. exigua* and the specialist *P. rapae* induced both aliphatic and indole GLS concentrations in the three *A. thaliana* ecotypes. Effects of herbivore feeding on the induction of GLS were more apparent for indole GLS than for aliphatic GLS, which is a general trend in GLS-containing plants that are attacked by herbivores (Mewis et al. 2006; Gols et al. 2008b; Textor and Gershenzon 2009). Feeding by *S. exigua* and *P. rapae* resulted in similar induction strength of GLS and in similar GLS profiles after induction.

Similar to what was observed for the *A. thaliana* ecotypes, herbivory also induced indole GLS concentrations in transformed Col-0-MYB28 plants. Unexpectedly, herbivore feeding did not induce aliphatic GLS in these transformed plants. Most likely, the physiological maximum production of aliphatic GLS by Col-0 had been reached by inserting the HAG1/MYB28 transcription factor behind a constitutive promoter.

7.4.2 Effects of GLS on the two herbivores

Both the generalist *S. exigua* and the specialist *P. rapae* performed poorest, in terms of slower development and smaller adults, on the *A. thaliana* ecotype with the highest aliphatic GLS concentrations and best on the *A. thaliana* ecotype with the lowest aliphatic GLS concentrations. Thus, aliphatic GLS concentrations seemed to be important in determining performance of both the generalist and the specialist herbivore. As expected, survival was much lower and the difference in performance among the ecotypes was larger for the generalist *S. exigua* than for the specialist *P. rapae*. *Pieris rapae* possesses a nitrile-specifier protein (NSP) that diverts GLS degradation from toxic isothiocyanates to less toxic nitriles (Wittstock et al. 2004). However, despite the NSP, a negative correlation between GLS concentrations and the performance of *P. rapae* was observed. There are several potential explanations for this negative effect. *Pieris rapae* excretes isothiocyanates in the faeces (Agelopoulos et al. 1995). This suggests that *P. rapae* cannot completely prevent formation of isothiocyanates by the activity of the NSP. Furthermore, the NSP is probably not equally efficient in inhibiting the breakdown all GLS compounds into toxic products (Gols et al. 2008b; H. Vogel personal communication). Moreover, although there is not much known about the toxicity of GLS hydrolysis products other than isothiocyanates to insects, the nitriles that are produced by the activity of the NSP might still be toxic to *P. rapae* (Burow and Wittstock 2009; Hopkins et al. 2009). Our results are in agreement with other studies that found negative correlations between concentrations of aliphatic GLS and their breakdown products for the generalist *S. exigua* (Gigolashvili et al. 2007; Arany et al. 2008; Müller et al. 2010) and the specialist *P. rapae* (Agrawal and Kurashige 2003). However, opposite to our findings, other studies did not find major changes in larval development of *P. rapae* due to increased aliphatic GLS concentrations (Gols et al. 2008b; Müller et al. 2010). Perhaps the difference between the studies in the strain of *P. rapae* or the plant species that was used resulted in a differential effect of aliphatic GLS on *P. rapae* performance.

To experimentally test whether aliphatic GLS affect the performance of the two herbivores, herbivore performance on Col-0 and Col-0-MYB28 aliphatic GLS-overexpressing plants was compared. As expected, performance of both herbivores, in terms of survival, development and adult size, was lower on Col-0-MYB28 plants than on Col-0 plants. However, these negative effects were only statistically significant for *P. rapae*, probably due to

the low number of surviving *S. exigua* adults on Col-0-MYB28 plants. Although herbivore-infested Col-0-MYB28 plants contained slightly higher concentrations of aliphatic GLS than herbivore-infested Col-0 plants, this difference was not statistically significant. It has to be noted that GLS concentrations of the plants were analysed after feeding by herbivores for five days, whereas the herbivores in the performance experiment were transferred to uninfested plants as neonates. Due to induction effects, GLS concentrations of Col-0 plants were probably higher after five days of feeding than when the neonates were transferred to the uninfested plant. Furthermore, the herbivores in the performance experiment received an additional uninfested plant if the previous plant was completely consumed, which happened on average one or two times for each larva (data not shown). Because uninfested Col-0-MYB28 plants contained higher aliphatic GLS concentrations than uninfested Col-0 plants, it is expected that the herbivores were exposed to these higher GLS concentrations at least at the beginning of their development and every time they received an additional plant, and that this negatively affected their performance. Although we cannot rule out that the transformation of Col-0 affected other plant traits besides the production of aliphatic GLS, we have no indications for this, as there were no differences in several plant traits such as biomass, diameter and trichome density between Col-0 and Col-0-MYB28 plants (data not shown).

Because the *A. thaliana* ecotypes/lines did not differ in indole GLS, effects of indole GLS on the performance of *S. exigua* and *P. rapae* could not be tested. Negative correlations between indole GLS concentrations and performance of *S. exigua* (Müller et al. 2010) and *P. rapae* (Gols et al. 2008a; Gols et al. 2008b; Müller et al. 2010) have been reported before.

Performance of the herbivores might not have been affected only by total aliphatic or indole GLS concentrations, but also by specific GLS. The multivariate analysis mostly separated the GLS profile of Cvi plants from the profile of the other two ecotypes, which corresponded to the largest difference in herbivore performance. It has been proposed that specific GLS can shape insect performance and abundance more strongly than total concentrations of these compounds (Poelman et al. 2009b; Kos et al. 2011a), and that plants may maintain variation in their chemical profile to confer resistance to many different attackers (Jones and Firn 1991; Newton et al. 2009b). As expected, Col-0 and Col-0-MYB28 plants did not show qualitative differences in GLS profiles, but only quantitative differences, *i.e.* differences in concentrations of

each of the produced compounds.

7.4.3 Effects of GLS on the third trophic level

The percentage of successful parasitism of *P. rapae* by the parasitoid wasp *H. ebeninus* was affected by the *A. thaliana* ecotype that its host developed on. Interestingly, the percentage of successful parasitism was highest on the ecotype on which the performance of the host was lowest, and *vice versa*, although not all pair-wise differences were statistically significant. Larvae of *P. rapae* have an immune system that enables them to encapsulate the eggs of their parasitoids, leading to egg death. Larval weight of *P. rapae* correlates positively with encapsulation rates and the strength of induced plant defences correlates negatively with encapsulation rates (Bukovinszky et al. 2009). Perhaps the larger *P. rapae* larvae that developed on the ecotype Eri had higher parasitoid egg encapsulation rates, leading to a lower percentage of successful parasitism, than the smaller larvae that developed on Cvi. However, encapsulation rates of *H. ebeninus* eggs by *P. rapae* were not quantified. Negative correlations between host size and the percentage of successful parasitism were not observed when comparing Col-0 and Col-0-MYB28 plants, even though there was a difference in *P. rapae weight* between both plant lines.

In contrast to the herbivores, development time and adult weight of *H. ebeninus* was not affected by the host plant ecotype/line. This is in agreement with the hypothesis that adverse effects of secondary metabolites on insect performance are often less pronounced in the parasitoid than in the herbivore (Gols and Harvey 2009). However, it is unclear whether the *H. ebeninus* larvae were actually exposed to GLS or their hydrolysis products. Direct effects of GLS and their hydrolysis products on the performance of parasitoids have never been studied, and it is not known whether parasitoids have the ability to detoxify GLS or their hydrolysis products (Gols and Harvey 2009). Effects of GLS on the behaviour of parasitoids have been studied before, and volatile breakdown products of GLS have been shown to attract several specialist parasitoids of herbivores that feed on GLS-containing plants (Bradburne and Mithen 2000; Blande et al. 2007; Mumm et al. 2008). Whether this is also true for *H. ebeninus* is presently not known.

7.4.4 Conclusion

This study shows that secondary plant metabolites can affect the performance

of insects at the second as well as the third trophic level. Aliphatic GLS concentrations were negatively correlated with several performance parameters of a generalist herbivore species that is not adapted to feeding on plants that contain these secondary metabolites, as well as a specialist herbivore species that is adapted to feeding on GLS-containing plants. This suggests that, despite the NSP that the specialist herbivore possesses, this herbivore cannot completely prevent formation of toxic GLS hydrolysis products. Perhaps brassicaceous plants have evolved ways to circumvent the herbivore's mechanism of diverting the breakdown of GLS into toxic products by increasing the production of specific GLS compounds that these herbivores cannot detoxify. The host plant ecotype did not affect only the performance of the herbivores, but also the performance of a parasitoid of one of these herbivores. Surprisingly, the percentage of successful parasitism was highest when the host developed on plants with the highest aliphatic GLS concentrations, possibly caused by negative effects on host immune responses.

Our study indicates that effects of GLS on herbivores can cascade up the food web and, through changes in host quality or constraints on immune responses, can positively affect parasitoids feeding on these herbivores. As a result, GLS can not only directly confer resistance against attacking herbivores by negatively affecting their performance, but also indirectly by increasing the performance of the parasitoids of these herbivores. It should be tested whether *H. ebeninus* is attracted to volatile breakdown products of GLS, which would further enhance the potential of GLS to confer resistance against herbivores in this study system.

7.5 Acknowledgements

We thank Prof. Flügge (University of Cologne, Germany) for providing the expression clone and Dr. Beekwilder for communications with Prof. Flügge; Erik Poelman, Léon Westerd, André Gidding and Frans van Aggelen for rearing the insects; Ana Pineda and Rieta Gols for practical advice and Unifarm for rearing of the Brussels Sprouts plants. This work was supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (grant number 838.06.010).

7

8

Genetic engineering of plant volatile terpenoids: effects on a herbivore, a predator and a parasitoid

Martine Kos, Benyamin Houshyani, Aart-Jan Overeem, Harro J. Bouwmeester, Berhane T. Weldegergis, Joop J.A. van Loon, Marcel Dicke and Louise E.M. Vet

Submitted

Abstract

Transgenic plants that enhance the effectiveness of natural enemies can be developed by genetic engineering of the biosynthesis of volatile organic compounds (VOCs). Before the commercialisation of such transgenic plants can be pursued, detailed fundamental studies on their effects on herbivores and their natural enemies are necessary.

We constitutively expressed the linalool/nerolidol synthase gene *FaNES1* from strawberry in three *A. thaliana* ecotypes. We tested the behaviour of the aphid *Brevicoryne brassicae*, the parasitoid *Diaeretiella rapae* and the predator *Episyrphus balteatus* exposed to the transgenic plants and their isogenic ecotypes.

Transgenic *FaNES1*-expressing plants emitted (*E*)-nerolidol and larger amounts of (*E*)-DMNT and linalool. *Brevicoryne brassicae* was repelled by the transgenic lines of two of the ecotypes, whereas its performance was not affected. *Diaeretiella rapae* preferred aphid-infested transgenic plants over aphid-infested wild-type plants for two of the ecotypes. In contrast, female *E. balteatus* predators did not differentiate between aphid-infested transgenic or wild-type plants.

Our study suggests that genetically engineering plants to modify their emission of VOCs holds considerable promise for improving control of herbivores. However, our results have to be validated for a crop species before transgenic plants with enhanced attraction of natural enemies can provide interesting new components for Integrated Pest Management.

8.1 Introduction

During the last decades, the demand for environmentally friendly pest control in agriculture has increased. To meet this demand, Integrated Pest Management (IPM) has been developed as an approach that integrates *e.g.* breeding for host plant resistance, biological and cultural control; including a chemical control component only as a last resort (van Lenteren 1993; Dent 1995; Koul et al. 2004). Recently, it was suggested that insect-resistant transgenic crops may become vital components of IPM (Romeis et al. 2008a; Kos et al. 2009). At present, all commercially available insect-resistant transgenic crops employ direct resistance, and express genes coding for proteins naturally occurring in *Bacillus thuringiensis* (*Bt*) that are lethal for target herbivores (Aronson and Shai 2001; Chen et al. 2008). In contrast, indirect resistance, which influences the effectiveness of natural enemies of herbivores (Karban and Baldwin 1997; Dicke and Baldwin 2010), has been largely neglected as a trait amenable to transgenesis.

Herbivore damage induces the emission of volatile organic compounds (VOCs) that have been shown to attract natural enemies of herbivores (Vet and Dicke 1992; Heil 2008; Dicke and Baldwin 2010; Mumm and Dicke 2010). Transgenic plants that enhance the effectiveness of natural enemies can be developed by using genetic engineering of VOC biosynthesis (Bouwmeester et al. 2003; Degenhardt et al. 2003; Poppy and Sutherland 2004; Turlings and Ton 2006). There are several benefits of incorporating transgenic crops that enhance biological control into IPM. For example, pest resistance to a biological control agent is not likely to evolve (Bale et al. 2008) and the VOCs that transgenic plants emit can repel herbivores (Dicke 1986; Heil 2004; Sanchez-Hernandez et al. 2006; Yang 2008). However, there may also be ecological risks involved in using these transgenic plants, because the modified emission of VOCs might not only repel certain herbivores, it might also attract others (Carroll et al. 2006; Halitschke et al. 2008). Therefore, before transgenic crops with enhanced indirect resistance are implemented, information on the effects of the modification on (potential) pest herbivores is necessary.

Transgenic crops exhibiting enhanced attraction of natural enemies by the emission of novel VOCs or enhanced emission of native VOCs are not yet commercially available. Before commercialisation of these transgenic plants can be pursued, detailed fundamental studies on their effects on different groups of herbivores and their natural enemies are necessary. In the last

decade, a few laboratory and field studies have been performed, mostly with the model plant *Arabidopsis thaliana*. These studies show that the novel or enhanced emission of VOCs by genetic engineering can increase the attraction of predatory mites (Kappers et al. 2005), parasitoid wasps (Beale et al. 2006; Schnee et al. 2006) and entomopathogenic nematodes (in maize) (Degenhardt et al. 2003; Degenhardt et al. 2009), and can repel aphids (Aharoni et al. 2003) and moths (Yang 2008). However, comprehensive studies of the effects of transgenic plants with modified VOC emission on different functional groups of natural enemies simultaneously, as well as on their host or prey species, are needed.

The objectives of this study were to test the effects of transgenic *A. thaliana* plants with modified VOC emission on the behaviour of the specialist cabbage aphid *Brevicoryne brassicae*, the aphid parasitoid *Diaeretiella rapae* and the aphid predator *Episyrphus balteatus*. The model plant *A. thaliana* is ideally suited for testing effects of genetic engineering because it is easily transformed and has a short lifecycle (Aharoni et al. 2003). We constitutively expressed the linalool/nerolidol synthase gene *FaNES1* from strawberry in *A. thaliana* and targeted the enzyme to the mitochondria, which has been shown to result in the emission of two common herbivore-induced plant volatiles: the sesquiterpene (*E*)-nerolidol and its derivative, the homoterpene (*E*)-DMNT (4,8 -dimethylnona-l,3,7-triene) (Kappers et al. 2005). Furthermore, because FaNES1 is a dual-function enzyme that catalyses the formation of both nerolidol from FDP (farnesyl diphosphate) and linalool from GDP (geranyl diphosphate) with equal efficiency (Aharoni et al. 2005), the introduction of this enzyme in *A. thaliana* can also lead to emission of the monoterpene linalool if GDP is present (Aharoni et al. 2003). The modified VOC emission by transgenic *FaNES1*-expressing plants resulted in the attraction of predatory mites to these transgenic plants (Kappers et al. 2005), but other natural enemies have so far not been studied. In the literature, the effects of the manipulation of terpenoid biosynthesis in *A. thaliana* on insects are studied almost exclusively in the ecotype Col-0 (Aharoni et al. 2003; Kappers et al. 2005; Yang 2008). Our study employed a novel approach of using different *A. thaliana* ecotypes as background for the transformation and testing the effects of the transformation on the emission of transgene-related products and subsequently on the behavioural response of aphids, parasitoids and predators.

8.2 Materials and methods

8.2.1 Plant material and growth conditions

Three *Arabidopsis thaliana* (L.) Heynh. ecotypes were selected, based on their maximal divergence in metabolite profiles (Houshyani et al. 2012): Antwerpen (An)-1 (obtained from the European Arabidopsis Stock Centre (http:// nasc.nott.ac.uk/; An-1 = N944), Columbia (Col)-0 (provided by Dr. P. Reymond, Lausanne, Switzerland), and Eringsboda (Eri; collected in Sweden by members of the Laboratory of Genetics, Wageningen University; Eri-1 = CS22548).

Arabidopsis thaliana seeds were surface-sterilized overnight by a vapour-phase sterilization method. Hereto, the seeds were placed in a desiccator containing a mixture of 3 ml hydrochloric acid (37%, Merck KGaA, Darmstadt, Germany) and 100 ml bleach. Subsequentially, the seeds were inoculated on MS medium (purified agar 0.8% + 2.2 g L^{-1} 0.5 MS + vitamins; pH 6; 35 µg ml^{-1} kanamycin was added to the medium used for the T2 transgenic lines). After four days of stratification at 4 °C, plates were transferred to a growth chamber at 21 ± 2 °C, 50-70% relative humidity (RH) and a 8:16 light:dark (L:D) photoregime (with a light intensity of 200 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD)).

Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diameter) containing autoclaved soil (80 °C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times a week and the soil was treated weekly with entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) to control infestation by larvae of sciarid flies. Plants used in the experiments were six to seven weeks old and remained in the vegetative state during the experiments.

8.2.2 Transgenic *A. thaliana* plants

Constructs for transformation and transgenic plants were generated as described in Houshyani et al. (in prep.). The strawberry linalool/nerolidol synthase gene *FaNES1* and the endogenous gene farnesyl diphosphate synthase (*FPS2*) were constitutively expressed using the 35S CaMV promotor and the enzymes were targeted to the mitochondria of *A. thaliana* (Houshyani et al., in prep.). *FPS2* was overexpressed to ensure that plants produced enough precursor for (*E*)-nerolidol biosynthesis. Method S8.1 in the Supporting Information provides a detailed description of the generation of transgenic

FaNES1-expressing plants. T1 generation seedlings were selected on kanamycin plates (purified agar 0.8% + 2.2 g L^{-1} 0.5 MS + vitamins; pH 6; 50 µg ml^{-1} kanamycin) and the effectiveness of transformation was confirmed by gene-specific PCR. T2 generation seedlings of one positive line per ecotype (hereafter named An-FaNES1, Col-FaNES1 and Eri-FaNES1) were used in the experiments.

8.2.3 Insect rearing

Brevicoryne brassicae L. (Hemiptera: Aphididae) was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) in a greenhouse compartment.

Diaeretiella rapae McIntosh (Hymenoptera: Braconidae) was reared in gauze cages (30 x 40 x 60 cm) containing *B. brassicae*-infested *B. oleracea* plants in a climate chamber. Wasps were provided with water and honey. The *B. brassicae* and *D. rapae* cultures originated from individuals collected on *B. oleracea* in the vicinity of Wageningen (The Netherlands) in 2009.

Episyrphus balteatus de Geer (Diptera: Syrphidae) pupae were provided by Koppert Biological Systems and kept in gauze cages (67 x 50 x 67 cm) in a greenhouse compartment. Adults emerging from the pupae were provided with water, a *B. brassicae*-infested plant, organic sugar grains and bee-collected pollen provided by Koppert Biological Systems. All insect species were reared at 22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime.

8.2.4 Chemical and morphological plant traits

8.2.4.1 Plant morphology

Of ten plants per *A. thaliana* line, we measured foliar biomass (fresh weight), plant diameter and the number of leaves and quantified trichome density by counting the number of trichomes in a 25 mm^2 area in the central part of the abaxial side of one mature leaf using a stereomicroscope (Leitz Dialux 20 EB, Wetzlar, Germany; magnification 40x).

8.2.4.2 Dynamic headspace collection of aphid-infested plants

While testing the preference of *D. rapae* in a Y-tube olfactometer (see below), we simultaneously collected the headspace of the plants. Volatiles were collected by sucking air out of each cuvette at a rate of 90 ml min^{-1} for 3 h through a stainless steel cartridge (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, Illinois, USA). The fresh

weight of the plant foliage in each cuvette was measured after volatile collection. For each *A. thaliana* line, four to six replicates, each consisting of the headspace of four plants, were collected.

The headspace samples were analysed using a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) quadrupole mass spectrometer (MS) (see Method S6.2 in **Chapter 6** for a detailed description of the analytical method and the identification of the volatile compounds). The peak areas of all compounds were expressed per unit plant fresh weight. In the GC-MS and statistical analysis, we particularly focussed on the emission of the transgene-related products (*E*)-nerolidol, (*E*)-DMNT, and linalool.

8.2.5 Aphid performance

We tested whether aphid performance differed between the transgenic and wild-type line of each ecotype, because this might (partly) explain the behaviour of the aphid's natural enemies. Aphid performance was assessed in a climate chamber at 21 ± 2 °C, 50-70% RH and a 8:16 h L:D photoregime. Individual plants with insects were confined to cylindrical plastic containers (height 13 cm; diameter 11 cm) with a gauze lid. The light intensity at plant level was 200 µmol $m^{-2} s^{-1}$ PPFD. Plants were watered once a week. Several six-week-old plants of each ecotype/line were inoculated with 10 adult aphids. After 24 h, the adult aphids were removed and the produced offspring was allowed to develop for three days until they reached the second instar (L2). Three L2 nymphs were transferred to each of 25 plants of the same line as on which these nymphs had been feeding before. Daily, survival of the nymphs was recorded per plant until the first aphid on that plant reached the adult stage. The fastest developing adult was kept on the plant, while the other adults were removed. Any alate (winged) adults (ca. 5% of all adults) were excluded from the experiment. The development time until first reproduction (=T_d) of the remaining adult was recorded and the adult fresh weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany). The adult was allowed to feed on the plant and produce offspring, and after a certain number of days (equivalent to T_d), the number of offspring (=N) produced by the adult was counted. The estimated intrinsic rate of increase (r_m) was calculated for each aphid using the formula: $r_m = 0.738 \times (\ln N)/T_d$ (Karley et al. 2002).

8.2.6 Aphid preference

The preference of *B. brassicae* for the transgenic or the wild-type line of each ecotype was tested in two-choice bioassays in a greenhouse compartment (22 ± 2 °C, 60-70% RH and a 16:8 h L:D photoregime). One transgenic and one wild-type plant were connected by a paper bridge (2 x 3 cm). Ten *B. brassicae* adults from the stock rearing were released in the centre of the bridge and were allowed to walk towards and feed on the plants. After 24 h the number of aphids on both plants was counted. For each ecotype at least 22 replicates were performed (An-1: 38, Col-0: 22, Eri: 30).

8.2.7 Parasitoid and predator preference

The preference of parasitoids and predators for volatiles from the transgenic or the wild-type line of each ecotype was tested in two-choice bioassays. Six-to-seven week-old *A. thaliana* plants were infested with 100 *B. brassicae* nymphs of mixed instars three days prior to the bioassays.

8.2.7.1 Parasitoid preference for aphid-induced plant volatiles

Parasitoid behaviour was assessed in a Y-tube olfactometer in a climatized room at 22 ± 2 °C as described by Bukovinszky et al. (2005). Compressed air was filtered over charcoal and split into two air streams each at a flow of 2 L min^{-1}. Each air stream was led through a 5 L glass cuvette that contained four aphid-infested plants of either the transgenic or the wild-type line of an ecotype. Each air stream was then led into one of the two arms of the Y-tube. The olfactometer was illuminated from above using artificial light at an intensity of 60 µmol m^{-2} s^{-1} PPFD.

Naïve, mated two-day-old *D. rapae* females were allowed to oviposit for one h in aphids feeding on either the transgenic or the wild-type line of an ecotype (equally divided among the tested wasps) to increase their motivation to search for a host. Immediately after the training period, experienced wasps were individually released at the base of the Y-tube using a glass vial and their preference for one of both odour sources was recorded. A choice was recorded when a wasp crossed a finish line drawn one cm before the end of each arm, and did not return to the junction within 15 s. Wasps that did not move into one of the arms within 10 min or did not make a final choice within 15 min were considered as non-responsive and were omitted from the statistical analysis. Seven to nine new sets of transgenic and wild-type plants were used for each ecotype (An-1: 9; Col-0: 7; Eri: 7). For every new set of

plants, 20 wasps were tested. After every ten wasps, the position of the odour sources was exchanged to compensate for any asymmetry in the set-up.

8.2.7.2 Predator oviposition preference

Mated female hoverflies from the stock rearing were used in the behavioural assays when they were two to three weeks old. Females were transferred to a plastic cage (30 x 30 x 30 cm) containing one transgenic plant and wild-type plant of the same ecotype, and 10% sugar solution in the same greenhouse compartment as described under *Aphid preference*. Females were allowed to oviposit on the plants. After 24 h, the number of eggs deposited on each plant was counted. Females that did not lay any eggs (about 30% of the females) were eliminated from the analysis. For each ecotype, at least 23 replicates with ovipositing females were obtained (An-1: 29, Col-0: 33, Eri: 23).

8.2.8 Statistical analysis

For most analyses, the transgenic line was compared with the wild-type line for each ecotype. Analyses were performed in SPSS for Windows (18th edition, Chicago, Illinois, USA), unless indicated otherwise. If variables were log-transformed to obtain normality and equal variance, this is indicated in the relevant table of the *Results* section. To test the effect of the line (transgenic or wild-type) on the measured variables, we used Student's t-tests. If assumptions on normality and equal variance were violated, Mann-Whitney U tests were used. Aphid survival was averaged per plant and differences in aphid survival between the transgenic and wild-type line of each ecotype were analysed by logistic regression in GenStat (13^{h} edition, VSN International, UK; dispersion estimated). T-probabilities were calculated to test pair-wise differences between means. Differences in the emission of transgene-related VOCs a) among the three transgenic lines and b) among the three wild-type lines were tested with ANOVA and post-hoc Tukey tests on the log-transformed data.

Aphid and parasitoid preference, measured by the number of aphids or wasps choosing for either the transgenic or the wild-type line of each ecotype, was analysed using a Chi-square test, with the null-hypothesis that the aphids or wasps did not have a preference for any of the two lines. Effects of parasitoid experience on the preference of the wasps was tested by logistic regression (dispersion estimated) in GenStat. Predator preference, measured by the number of predator eggs on either the transgenic or the wild-type line of

each ecotype, was analysed with Wilcoxon's matched-pairs signed-rank test.

To test if there were differences in the entire volatile profile between the transgenic line and the wild-type line of each ecotype, we used multivariate discriminant analysis Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12^{th} edition, Umetrics, Umeå, Sweden) (Eriksson et al. 2006). To pre-process data, volatile amounts were log-transformed, mean-centred and scaled to unit variance.

8.3 Results

8.3.1 Plant morphology

Transgenic An-FaNES1 plants had a higher biomass but a similar plant diameter compared to the An-1 wild-type plants, whereas Col-FaNES1 and Eri -FaNES1 plants had a lower biomass and smaller diameter than their respective wild-type plants (Student's t-test, n = 10, biomass: An-1: t = -3.04, P = 0.007; Col-0: t = 6.38, P < 0.001; Eri: t = 3.49, P = 0.003; diameter: An-1: t = 1.40, P = 0.179; Col-0: t = 9.24, P < 0.001; Eri: t = 5.69, P < 0.001; Table 8.1). Plants of all transgenic lines had a larger number of leaves than plants of the wild-type lines, although this was only significant for ecotypes An-1 and Col-0 (Mann-Whitney U-test, n = 10, An-1: U = 7.50, P < 0.001; Col-0: U = 21.00, P

Table 8.1. Mean (± SE) morphological plant characteristics of wild-type and transgenic *FaNES1*-expressing lines of three *Arabidopsis thaliana* ecotypes

	***A. thaliana* line**					
Characteristic	**An-1**	**An-FaNES1**	**Col-0**	**Col-FaNES1**	**Eri**	**Eri-FaNES1**
Biomass (fresh weight; g)[a]	0.51 ± 0.02	0.63 ± 0.04 *	0.49 ± 0.02	0.36 ± 0.01 *	0.62 ± 0.05	0.43 ± 0.03 *
Diameter (cm)[a]	8.0 ± 0.1	7.6 ± 0.2 **ns**	8.2 ± 0.1	6.1 ± 0.2 *	8.7 ± 0.2	7.2 ± 0.2 *
Number of leaves[b]	21.5 ± 0.3	25.8 ± 1.1 *	22.1 ± 0.4	26.3 ± 1.6 *	26.7 ± 0.9	28.8 ± 2.1 **ns**
Trichome density[a,c]	8.1 ± 0.6	10.7 ± 0.8 *	13.7 ± 0.8	18.5 ± 1.2 *	6.4 ± 0.3	7.8 ± 0.6 *

*n = 10. [a] Analysed by Student's t-tests. [b] Analysed by Mann-Whitney U-tests. [c] Trichome density is the number of trichomes per 25 mm^2 leaf area of mature leaves. * denotes significant difference (P<0.05) and ns denotes no significant difference (P>0.05) between the wild-type and transgenic line for each ecotype.*

= 0.029; Eri: U = 44.50, P = 0.684; Table 8.1). For all ecotypes, leaves of transgenic *FaNES1*-expressing plants had a higher trichome density than leaves of wild-type plants (Student's t-test, n = 10, An-1: t = -2.74, P = 0.013; Col-0: t = -3.49, P = 0.003; Eri: t = -2.28, P = 0.035; Table 8.1).

8.3.2 Dynamic headspace collection of aphid-infested plants

Transgenic *FaNES1*-expressing lines of the three ecotypes emitted (*E*)-nerolidol, whereas wild-type plants of the three ecotypes did not emit this compound (Fig. 8.1). An-FaNES1 plants emitted larger amounts of (*E*)-nerolidol than Eri-FaNES1 plants (ANOVA, $F_{2,11}$ = 6.01; P = 0.017).

The transgenic *FaNES1*-expressing lines emitted larger amounts of (*E*)-DMNT than the corresponding wild-type plants, but the difference was only significant for ecotypes An-1 and Col-0 (Student's t-test, An-1: t = -3.38; P = 0.019; Col-0: t = -4.06; P = 0.007; Eri: t = -1.65; P = 0.194; Fig. 8.1). While (*E*)-DMNT was detected in all transgenic samples, it was detected in only two of the six wild-type An-1 samples, one of the four wild-type Col-0 samples and two of the four wild-type Eri samples. Col-FaNES1 plants emitted larger amounts of (*E*)-DMNT than Eri-FaNES1 plants (ANOVA, $F_{2,11}$ = 4.26; P = 0.043). The wild-type lines did not differ in emission of (*E*)-DMNT (ANOVA, $F_{2,11}$ = 0.295; P = 0.750).

For each ecotype, the transgenic *FaNES1*-expressing line emitted larger amounts of linalool than the wild-type plants, but the difference was only significant for ecotype An-1, due to the high variation in emission of this compound among replicates (Student's t-test, An-1: t = -3.55; P = 0.015; Col-0 and Eri: P > 0.05; Fig. 8.1). In ca. 50% of the wild-type samples linalool was detected (three of the six An-1 samples, two of the four Col-0 samples and three of the four Eri samples), while this compound was detected in all the transgenic samples. The three transgenic lines did not differ in the emission of linalool, neither did the three wild-type lines (ANOVA, P > 0.05 for both analyses).

Apart from the reported differences in the emission of (*E*)-nerolidol, (*E*) -DMNT and linalool, there were no differences in the overall volatile profile between aphid-infested plants of the transgenic and wild-type line of each ecotype, as analysed by a multivariate discriminant analysis using all other volatile compounds (no significant PLS-DA components could be extracted; see Table S8.1 in the Supporting Information for an overview of the volatile compounds detected in the headspace of the aphid-infested plants).

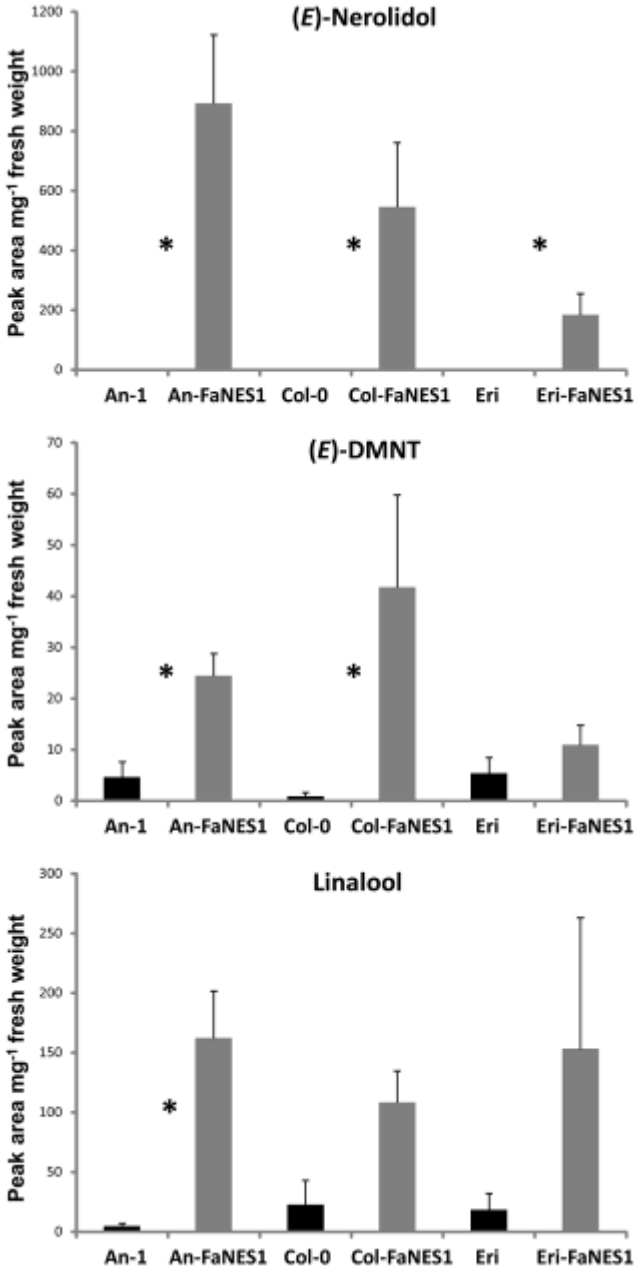


Fig. 8.1 *Emission of (E)-nerolidol, (E)-DMNT and linalool (peak area mg^{-1} fresh weight + SE) by aphid-infested wild-type or transgenic Arabidopsis thaliana lines of three ecotypes (An-1, Col-0 and Eri). Of each ecotype, a transgenic line (FaNES1) was created. n = 4-6 for each bar. An asterisk indicates a significant difference (P<0.05) in the emission of the compound between the wild-type and the transgenic FaNES1-expressing line for each ecotype, as analysed by Student's t-tests.*

8.3.3 Aphid performance

There were no differences in any of the aphid-performance parameters between transgenic and wild-type plants for each of the ecotypes ($P > 0.05$ for every comparison; Table 8.2).

8.3.4 Aphid preference

Aphids preferred wild-type Col-0 and Eri plants over plants of the corresponding transgenic line, but did not differentiate between An-1 and An-FaNES1 plants (Chi-square test, An-1: $\chi^2 < 0.01$, $P = 1.000$; Col-0: $\chi^2 = 10.47$, $P = 0.001$; Eri: $\chi^2 = 4.27$, $P = 0.039$; Fig. 8.2).

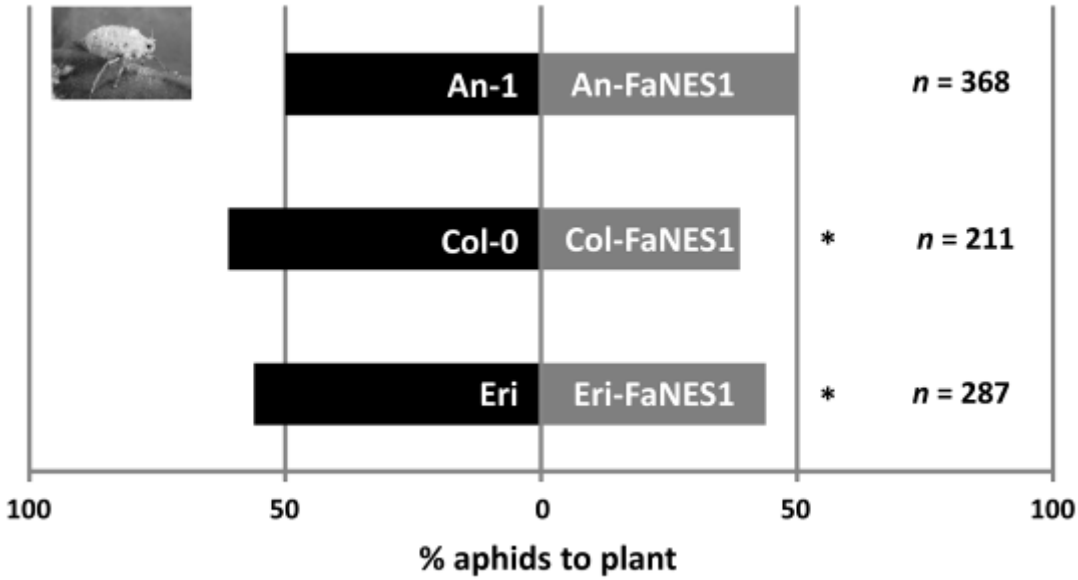


Fig. 8.2 *Preference of Brevicoryne brassicae adults in two-choice tests for the wild-type or transgenic line of three Arabidopsis thaliana ecotypes (An-1, Col-0 or Eri). Of each ecotype, a transgenic line with enhanced emission of volatiles (FaNES1) was created. Each bar represents the percentage of aphids that made a choice for the indicated plant line. The total number of tested aphids is indicated on the right. An asterisk indicates a significant preference (P<0.05) for one of the two lines in a combination, as analysed by Chi-square tests. Photograph by Tibor Bukovinszky; www.bugsinthepicture.com.*

Table 8.2. Mean (± SE) performance characteristics of *Brevicoryne brassicae* on wild-type and transgenic *FaNES1*-expressing lines of three *Arabidopsis thaliana* ecotypes

	***A. thaliana* line**					
Performance parameter	**An-1**	**An-FaNES1**	**Col-0**	**Col-FaNES1**	**Eri**	**Eri-FaNES1**
Survival until adult stage (%)[a]	87	88 **ns**	93	89 **ns**	83	88 **ns**
Development time until first reproduction in days (*T*d)[b]	8.8 ± 0.1	8.8 ± 0.1 **ns**	9.7 ± 0.1	9.8 ± 0.1 **ns**	9.5 ± 0.1	9.6 ± 0.1 **ns**
Adult fresh weight in mg[c]	0.410 ± 0.017	0.417 ± 0.021 **ns**	0.329 ± 0.015	0.303 ± 0.011 **ns**	0.339 ± 0.011	0.320 ± 0.011 **ns**
Number of offspring (*N*) in time period equivalent to *T*d[c]	34.4 ± 3.4	34.0 ± 2.8 **ns**	24.8 ± 2.5	21.8 ± 2.2 **ns**	25.4 ± 2.0	23.0 ± 2.9 **ns**
Estimated intrinsic rate of population increase (r_m)[b]	0.288 ± 0.007	0.288 ± 0.008 **ns**	0.233 ± 0.009	0.217 ± 0.010 **ns**	0.246 ± 0.006	0.230 ± 0.008 **ns**

[a] Performance parameter was averaged per plant before statistical analysis and analysed by logistic regression and post-hoc T-probability tests. [b] Analysed by Mann-Whitney U-tests. [c] Performance parameter was log-transformed in statistical analysis to obtain normality and analysed by Student's t-tests. ns denotes no significant difference (P>0.05) in performance between aphids feeding on the wild-type and transgenic line for each ecotype

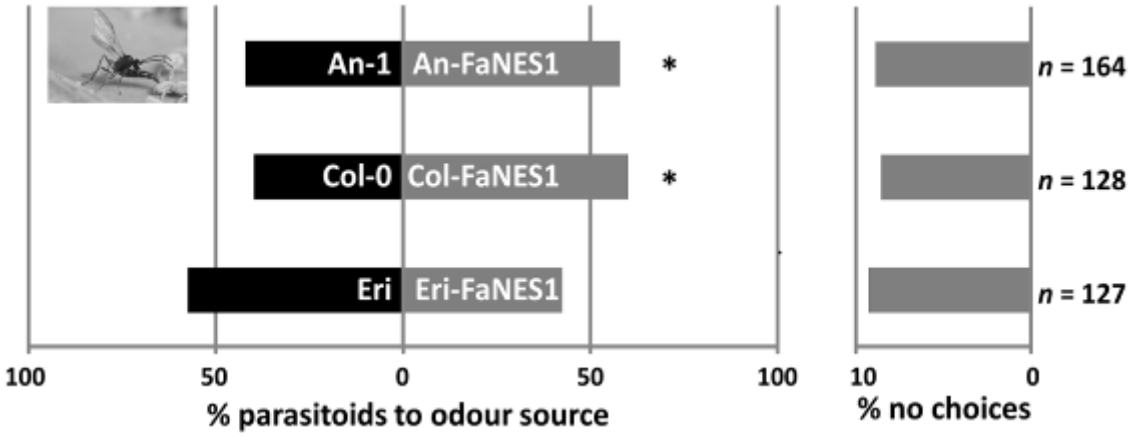


Fig. 8.3 *Responses of Diaeretiella rapae parasitoid females to volatile blends emitted by aphid-infested wild-type or transgenic Arabidopsis thaliana lines of three ecotypes (An-1, Col-0 and Eri) in a Y-tube olfactometer. Of each ecotype, a transgenic line with enhanced emission of volatiles (FaNES1) was created. Each bar represents the percentage of females that made a choice for the indicated odour sources. The percentage of parasitoids that did not make a choice ('% no choices') in each experiment and the total number of tested females is indicated on the right. An asterisk indicates a significant preference (P<0.05) for one of the two lines in a combination, as analysed by Chi-square tests. Photograph by Nina Fatouros; www.bugsinthepicture.com.*

8.3.5 Parasitoid preference for aphid-induced plant volatiles

Parasitoids preferred volatiles from aphid-infested transgenic An-FaNES1 and Col-FaNES1 lines over volatiles from the corresponding aphid-infested wild-type lines, but did not differentiate between volatiles from aphid-infested Eri and aphid-infested Eri-FaNES1 plants (Chi-square test, An-1: $\chi^2 = 4.12$, $P = 0.042$; Col-0: $\chi^2 = 5.28$, $P = 0.022$; Eri: $\chi^2 = 2.84$, $P = 0.092$; Fig. 8.3). There was no effect of previous oviposition experience on the preference of the wasps (logistic regression, $P > 0.05$ for every combination).

8.3.6 Predator oviposition preference

Female *E. balteatus* did not differentiate between aphid-infested plants of the transgenic or wild-type line of any of the ecotypes (Wilcoxon's matched-pairs signed-rank test, $P > 0.05$ for every comparison; Fig. 8.4).

8.4 Discussion

8.4.1 Volatile emission by transgenic plants

Aphid-infested transgenic *FaNES1*-expressing plants emitted the sesquiterpene (*E*)-nerolidol, whereas this compound was not emitted by aphid-infested wild-type plants. Aphid-infested transgenic plants also emitted the homoterpene (*E*)-DMNT. (*E*)-DMNT is synthesised from (*E*)-nerolidol by endogenous *Arabidopsis* enzymes (Kappers et al. 2005). The emission of (*E*)-nerolidol and (*E*)-DMNT from transgenic *FaNES1*-expressing plants is in agreement with the study by Kappers et al. (2005) on ecotype Col-0, in which the same *FaNES1*-construct was used, but without the *FPS2*-construct.

Transgenic *FaNES1*-expressing plants also emitted larger amounts of the monoterpene linalool compared to wild-type plants, although this was only significant for ecotype An-1. FaNES1 is a dual-function enzyme that catalyses the formation of both linalool from GDP and nerolidol from FDP with equal efficiency (Aharoni et al. 2005). Our finding of higher linalool emission by transgenic plants suggests that there is a small pool of GDP present in the mitochondria of the *A. thaliana* ecotypes used. However, our finding could also mean that there was leaking of the linalool/nerolidol synthase from the mitochondria of the transformants in our study, as Kappers et al. (2005) did not report the emission of linalool in transgenic *FaNES1*-expressing plants.

The detection of (*E*)-DMNT and linalool in some of the wild-type samples is remarkable, as most previous studies report that *A. thaliana* plants do not produce these compounds in the vegetative state (van Poecke et al. 2001; Aharoni et al. 2003; Kappers et al. 2005; Yang 2008). However, these latter studies were done using an older version mass spectrometer (van Poecke et al. 2001), or used solid-phase microextraction (SPME) GC-MS (Aharoni et al. 2003; Kappers et al. 2005; Yang 2008). Perhaps their methods were less sensitive for (*E*)-DMNT and linalool than the method we used. In our study, (*E*)-DMNT and linalool were not detected in all wild-type samples, suggesting that the amounts were below the detection limit of the GC-MS, and the amounts in the samples in which these compounds were actually detected were small. A more recent study using the same GC-MS set-up as we did also recorded (*E*)-DMNT and linalool (and even (*E*)-nerolidol) in vegetative plants of the three *A. thaliana* ecotypes that we studied (Snoeren et al. 2010), suggesting that *A. thaliana* is indeed able to produce these compounds in the rosette stage.

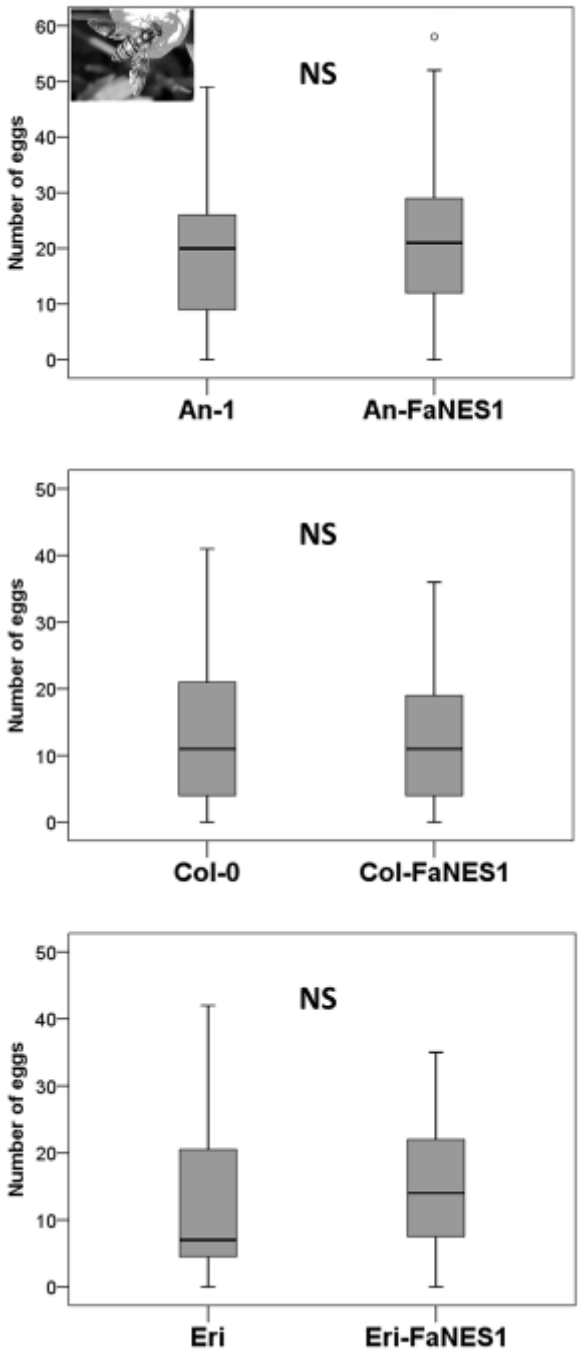


Fig. 8.4. *Oviposition preference (number of eggs) of the aphid predator Episyrphus balteatus in a two-choice assay with aphid-infested plants of wild-type and transgenic Arabidopsis thaliana lines of three ecotypes (An-1, Col-0 and Eri). Of each ecotype, a transgenic line with enhanced emission of volatiles (FaNES1) was created. The boxes span the first to third quartile range with the line across the box indicating the median. The whiskers represent the range. Open circles represent outliers. NS indicates a non-significant difference (P>0.05) between the number of eggs deposited on the wild-type and the transgenic line of each ecotype as analysed by the Wilcoxon's matched-pairs signed-rank test. Photograph by Han Endt; www.veluwe-insecten.nl/zweefvliegen/episyrphus/episyrphus.html.*

The level of emission of (*E*)-nerolidol and (*E*)-DMNT by the transgenic plants was dependent on the genetic background of the transgenic line. It can be expected that plants with a higher biomass emit larger amounts of volatiles. In agreement with this, An-FaNES1 plants emitted larger amounts of (*E*)-nerolidol than Eri-FaNES1 plants. However, the difference in biomass does not explain why Col-FaNES1 plants emitted larger amounts of (*E*)-DMNT than Eri-FaNES1 plants, as Col-FaNES1 plants had a lower biomass than Eri-FaNES1 plants. This suggests that the expression of the linalool/nerolidol synthase gene, the efficiency of the produced enzymes, or the availability of substrates differs among different ecotypes.

The biosynthesis of the novel terpenoids by transgenic *FaNES1*-expressing plants has been shown to impose costs on plant growth, and was speculated to be due to a reduction in the availability of substrates for other metabolites that play an important role in plant growth, or to direct toxic effects of the transgenic products to the plant (Aharoni et al. 2003; Kappers et al. 2005). In agreement with this, we observed growth retardation of transgenic plants for two of the three ecotypes (Col-0 and Eri). However, An-FaNES1 plants, which had the highest emission of (*E*)-nerolidol and linalool in their headspace, were actually larger than the wild-type An-1 plants. This suggests that the effects on plant growth are dependent on the genetic background of the transgenic line, or on the exact insertion position of the transgene in the genome.

Transgenic *FaNES1*-expressing plants of each ecotype exhibited a higher trichome density than the corresponding wild-type plants. This is probably due to the transgenic plants having a smaller diameter than the wild-type plants, suggesting that the leaves of the transgenic plants were smaller. It is known that trichomes are produced in the beginning of the development of a leaf and the growth of new trichomes is limited when the leaf starts to expand (Hülskamp and Schnittger 1998).

8.4.2 Aphid performance and preference

Performance of the aphid *B. brassicae* did not differ between transgenic and wild-type plants for any of the three ecotypes. This suggests that the transgenic and wild-type plants did not differ in quality for aphid development, even though the transgenic plants had higher trichome densities, and were smaller (for two of the three ecotypes). Transgenic *FaNES1*-expressing plants that emitted large amounts of linalool (because the enzyme was targeted to

the plastids, instead of the mitochondria) also did not affect the performance of caterpillars of the herbivore *Plutella xylostella* (Yang 2008).

Brevicoryne brassicae aphids preferred wild-type Col-0 and Eri plants over plants of the corresponding transgenic line, suggesting that the volatiles (*E*)-nerolidol, (*E*)-DMNT and/or linalool emitted by Col-FaNES1 and Eri-FaNES1 plants acted as repellents to *B. brassicae*. This is in agreement with the study by Aharoni et al. (2003), in which transgenic *FaNES1*-expressing plants with a high emission of linalool and linalool derivatives and a low emission of nerolidol repelled the aphid *Myzus persicae*. Linalool and/or nerolidol were also found to be repellent to spider mites, thrips, and moths (Dabrowski and Rodriguez 1971; Doskotch et al. 1980; Kessler and Baldwin 2001; Koschier et al. 2002; Yang 2008). However, repellence of linalool and/or nerolidol to *B. brassicae* does not explain why aphids did not differentiate between An-1 and An-FaNES1 plants, the latter producing the largest amounts of (*E*)-nerolidol and linalool of the three transgenic lines. We cannot rule out effects of plant biomass or trichome density or of an interaction between these variables and VOC emission on aphid preference.

8.4.3 Parasitoid preference

The aphid parasitoid *D. rapae* preferred plants of An-FaNES1 and Col-FaNES1 over the wild-type plants. *Diaeretiella rapae* parasitizes many aphid species on at least 16 plant species (Pike et al. 1999), which could explain why this parasitoid is attracted towards plants emitting (*E*)-nerolidol, (*E*)-DMNT and linalool, all three common herbivore-induced volatiles. (*E*)-nerolidol, the most dominant transgene-related product in the headspace of the transgenic plants, is often reported as a component of the volatile blend of herbivore-induced plants. However, to our knowledge, attraction of natural enemies of herbivores specifically towards a source emitting this compound in pure form has only been reported for predatory mites (Kappers et al. 2005). The attractiveness of (*E*)-DMNT and linalool to several species of natural enemies has often been reported (Dicke et al. 1990a; Dicke et al. 1990b; Du et al. 1998; Hoballah et al. 2002; de Boer et al. 2004; Gouinguene et al. 2005; Kappers et al. 2005). Parasitoids did not differentiate between Eri and Eri-FaNES1 plants, probably because Eri-FaNES1 plants emitted only small amounts of (*E*)-nerolidol and (*E*)-DMNT.

8.4.4 Predator preference

In contrast to the parasitoid wasp, females of the aphid predator *E. balteatus* did not differentiate between aphid-infested plants of the transgenic and wild-type line of each ecotype. This suggests that (*E*)-nerolidol, (*E*)-DMNT and linalool do not affect the behaviour of *E. balteatus* at the emission rates tested. *Episyrphus balteatus* mainly uses aphid-derived chemicals to locate its prey (Almohamad et al. 2009). There are unfortunately only few published studies on the role of volatiles in hoverfly attraction. Verheggen et al. (2008) recorded that the sesquiterpenes α-humulene and β-caryophyllene did not evoke electroantennographic (EAG) responses in *E. balteatus*, whereas the aphid alarm pheromone (*E*)-β-farnesene did evoke responses. Monoterpenes elicited only a low response, compared to the response to green leaf volatiles, and adding the monoterpene limonene to uninfested plants did not affect the oviposition behaviour of females (Verheggen et al. 2008). The low response to plant terpenoids might explain why *E. balteatus* did not differentiate between transgenic plants and wild-type plants in our study, which differed only in emission of three terpenoids.

In contrast to *D. rapae*, during the behavioural bioassays *E. balteatus* had access to the aphid-infested plants and could, therefore, also have used plant cues other than volatiles, as well as aphid cues, to select a plant for oviposition. We did not observe that hoverflies preferred the wild-type plants, which had lower trichome densities, over the transgenic plants, although it has been shown before that female hoverflies have difficulties in landing on plants with high trichome densities (Verheggen et al. 2009). Aphid body weight and population size did not differ between transgenic and wild-type plants, which may explain why hoverflies did not differentiate between the plants.

8.4.5 Potential of application of the transgenic approach in IPM

Our results indicate that genetically engineered plants with modified emission of VOCs can enhance the attraction of natural enemies of herbivores. Furthermore, a potential pest herbivore was repelled by the transgenic plant, while its performance was not affected, which increases the potential of these transgenic plants for pest control. Although our study suggests that genetically engineering plants to modify their emission of VOCs holds considerable promise for improving control of herbivores, it was performed with the model plant *A. thaliana*. Our results have to be validated with actual crop species before such a transgenic approach can be applied in IPM. The recent field

study by Degenhardt et al. (2009) is an excellent example of how this technique could be successfully applied in a crop plant.

Before implementation of transgenic plants with modified emission of VOCs in IPM can be pursued, the technique for creating these transgenic plants should be optimised. Similar to previous studies (Aharoni et al. 2003; Kappers et al. 2005; Degenhardt et al. 2009), we used a constitutive promotor, which results in the constitutive production of the transgene-related products. Replacement of the constitutive promotor by an inducible promotor, for example one that is induced by herbivore feeding, will be essential. Natural enemies are capable of learning, and if the VOCs are emitted constitutively, the natural enemies likely learn to ignore the signal if it is not associated with a host or prey (Papaj et al. 1994; Kos et al. 2009). Furthermore, by using an inducible promotor, negative effects of the constitutive production of the transgenic products on plant growth may be prevented (Yang 2008).

8.4.6 Conclusion

Our study shows that genetically transforming plants to modify the emission of terpenoids can repel a herbivore and enhance the attraction of one of its natural enemies, holding promise for improving control of herbivores in agriculture. However, our results have to be validated for a crop species before transgenic plants with enhanced attraction of natural enemies can provide interesting new components for IPM.

8.5 Acknowledgements

We thank Ana Pineda for practical advice; Qianjue Wang for help with the behavioural assays; Koppert Biological Systems for providing *E. balteatus* pupae and Unifarm for rearing of the Brussels sprouts plants. This work was supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (grant number 838.06.010).

9

Guidelines for the assessment of ecological effects of transgenic crops on non-target organisms

Martine Kos[*]**,** Benyamin Houshyani[*], Patrick Kabouw[*], Arjen Biere, Harro J. Bouwmeester, Nicole M. van Dam, Marcel Dicke, Joop J.A. van Loon, Wim H. van der Putten and Louise E.M. Vet

** Authors contributed equally to the manuscript*

To be submitted in a slightly different form to the Dutch Advisory Commission on Genetic Modification (COGEM)

9.1 Introduction

The main objective of the ERGO-project was to study the ecology of transgenic crops to be able to develop ecology-based guidelines for assessing the non-target effects of new transgenic crops (**Chapter 1**). This document aims to provide these guidelines and can be used when permission for field trials or commercialisation of a new transgenic crop is submitted by the breeding company that produced the transgenic crop (the applicant) to the Dutch Advisory Commission on Genetic Modification (COGEM). The guidelines address four main questions that are important in evaluating the non-target effects of newly developed transgenic crops: 1) Did the applicant provide data representing the baseline variation? 2) Did the applicant measure the traits under representative testing conditions? 3) Did the applicant test relevant organisms and traits? and 4) Did the applicant interpret the results correctly? A summarizing step-wise question list is added to facilitate the use of these guidelines by the expert advisors at COGEM (Supporting Information 9.1). This document is based on the results from three connected PhD-theses in which the plant family Brassicaceae was used as a model system (this thesis; Houshyani 2012; Kabouw 2012). Therefore, the guidelines have been validated for brassicaceous plant species, and most of the results that are discussed in this document were obtained from experiments with brassicaceous plants. Although the Brassicaceae family was used as a model system, this document has broader application possibilities than for brassicaceous species only.

9.2 Did the applicant provide data representing the baseline variation?

9.2.1 What is baseline variation?

Commonly, the assessment of non-target effects of a transgenic crop is done by comparing the transgenic line with the relevant isogenic line (*i.e.* the original genotype into which the transgene was introduced). However, a certain range of variation in the effects on non-target organisms also exists among conventional varieties of the crop species. This variation might be more extensive than between a transgenic line and its isogenic original line. Consequently, the observed effects of the transgenic plant may well fit within the variation that exists among conventional varieties. For this reason, it is necessary to first represent the 'baseline variation' in order to properly evaluate the ecological effects of a transgenic crop within the range of

variation existing within the crop species. In this document the term 'baseline variation' is defined as 'the variation in effects observed among a selection of non-transgenic varieties, across sets of environmental conditions'. The baseline variation refers to the natural variation in any plant trait, such as the metabolome of a plant, or to natural variation in the effects that these traits have on other organisms.

Information on baseline variation is necessary in order to assess whether the transgenic variety is disproportionally different from the varieties that were produced by traditional breeding. In *Brassica oleracea* L., for instance, centuries of breeding have resulted in a large number of varieties (cultivars). These varieties differ in the level of direct resistance, based *e.g.* on the biosynthesis of glucosinolates (GLS) (Kushad et al. 1999; Gols et al. 2008b; Poelman et al. 2009b; Kabouw et al. 2010b; Kos et al. 2011a) and indirect plant resistance, *i.e.* attraction of natural enemies (Chin and Lindsay 1993; Geervliet et al. 1997; Kalule and Wright 2004; Poelman et al. 2009a). The variation in effects on non-target organisms among these conventional cabbage varieties may potentially be larger than between a transgenic cabbage line and its isogenic line. Comparing the effects of the transgenic crop with the baseline variation in effects is, therefore, crucial in order to make realistic impact assessments of transgenic crops.

9.2.2 How to represent baseline variation?

The baseline variation can be represented by selecting a range of conventionally bred and/or wild-type genotypes/varieties that have a close (*i.e.* can be crossed with) taxonomic relationship at the species level with the transgenic plant. The environmental conditions under which a plant grows affect its interactions with other organisms. For example, soil characteristics do not only influence plant quality, but also both aboveground and belowground biota and the plant-mediated interactions between these biota (van der Putten et al. 2001; Bruinsma et al. 2003; Pineda et al. 2010; Kabouw et al. 2011). Therefore, the baseline variation should cover both genotypic variation and environmental variation. This environmental variation can refer to both the geographical zone, *e.g.* climate and soil type, and the management system, *e.g.* land use and cultivation practices (EFSA 2010; see also paragraph 9.3.3). Representation of the baseline variation must be based on proper knowledge of the conventional agro-ecosystem.

An important aspect for the selection of varieties constituting the baseline variation is determination of the relevant traits. This is, amongst others, dependent on the modification used in the transgenic crop. If the modification concerns enhanced resistance to herbivores, the varieties to be used for representation of the baseline variation can, for example, be selected on the basis of known (preferably experimentally assessed) varying levels of resistance to herbivores. If transgenic plants are generated by introducing heterologous genes, it is preferable to include into the baseline varieties spanning the complete range of the effects on herbivores (from very susceptible to very resistant ones). This applies, for instance, to transgenic plants containing genes coding for *Bacillus thuringiensis* (*Bt*)-toxins.

In addition, untargeted metabolomics analysis could be useful for representation of the baseline variation. The metabolome of plants (the complete set of primary and secondary metabolites) displays genetic variation and - on top of that - varies in response to changes in biotic and abiotic environmental conditions. Metabolomics can be used as a relatively quick tool to select genotypes/varieties and environmental conditions that will encompass as much possible variation, which can then be used in target and non-target studies (Houshyani et al. 2012). However, it is not possible to guarantee that the full range of variation has been covered, because the sample of genotypes/varieties included in the metabolomics analysis is, at present, necessarily limited.

9.3 Did the applicant measure the traits under representative testing conditions?

9.3.1 Tiered approach

Based on suggestions from the literature (Dutton et al. 2003; Poppy and Sutherland 2004; Andow and Zwahlen 2006; Birch et al. 2007; Charleston and Dicke 2008), a stepwise (tiered) approach for testing the non-target effects of transgenic crops is proposed (Fig. 2.2 in **Chapter 2**). In this tiered approach, the assessment increases in complexity and realism based on the knowledge that is gained in previous steps. Between the tiers, sufficient feedback is necessary rather than considering the tiers as steps in a sequential, linear approach. If necessary, results from one level could be re-examined at another level to fill certain knowledge gaps (Birch et al. 2007).

9.3.2 Predicting effects under field conditions from greenhouse data

The tiered approach starts with laboratory and greenhouse studies. The main reasons to conduct such studies are the containment of the transgenic plant and the relatively small costs of greenhouse studies. If greenhouse studies can be used to predict non-target effects in the field, this will greatly facilitate future ERAs for new transgenic crops. Several studies have compared results from the greenhouse with results from the field. For example, four white cabbage cultivars were tested in both greenhouse and field studies for their metabolite composition and their suitability for herbivores and their natural enemies. The profiles of root GLS, the characteristic secondary metabolites of brassicaceous plants, were generally comparable between greenhouse studies and field trials, whereas shoot GLS profiles were highly variable and not comparable (Kabouw et al. 2010a). For performance/abundance of aboveground herbivores and their natural enemies, a similar ranking of the cultivars for both greenhouse and field experiments was observed (Kabouw et al. 2011; Kos et al. 2011a; 2011b). It has also been found that field parasitism rates of caterpillars on these four cultivars were reliably predicted by the behaviour of the parasitoids in the greenhouse (Poelman et al. 2009a).

In another study the metabolome profiles of *Arabidopsis thaliana* plants grown in four different environments (a climate chamber in soil or hydroponics, a controlled-conditions greenhouse, an uncontrolled-conditions greenhouse) were compared. The interaction between ecotype and environment explained little of the variation among metabolite profiles, suggesting that the metabolic responses of the different ecotypes to changes in the environment were quite similar (Houshyani et al. 2012).

The cited studies show that greenhouse studies can often, but not always, be a good indicator for the effects that can be expected in the field. However, in the greenhouse, mostly single plant-organism interactions are tested, which might not be representative for the complexity of interactions in the field. For example, early season herbivores differentially affect the responses of plant resistance to subsequently colonising herbivores, and thereby differentially affect the abundance of herbivores that occur later in the growing season (Poelman et al. 2008a). Such effects cannot be tested in the greenhouse. Furthermore, in greenhouse studies often homogenised and sterilised potting soil is used that lacks the complex plant-soil interactions that can be present in the field. Thus, despite the fact that greenhouse studies are a good start and can save costs associated with field experiments in the

beginning of the risk assessment procedure, field studies are always required later in the risk assessment procedure to validate the results from the greenhouse.

9.3.3 Environmental conditions

Similar to what was described for the representation of the baseline variation, it is important to include several environmental growing conditions (*e.g.* soil type and climatic region) that are representative of the cultivation area of the crop when testing non-target effects. EFSA (European Food Safety Authority) advises applicants to identify the geographical regions within the EU where the transgenic plant is planned to be introduced, and to select several of these regions which reflect the appropriate climatic, ecological and agricultural conditions for testing effects of the transgenic crop on non-target organisms (EFSA 2010). Growing conditions that are not commercially used for a crop (*e.g.* hydroponics for maize) have a limited predictive value and could be excluded, because these might lead to a biased view on the non-target effects that might not be observed under 'normal' cultivation conditions.

9.4 Did the applicant test relevant organisms and traits?

9.4.1 Selection of non-target organisms

There are several publications that suggest criteria for selecting aboveground and belowground non-target organisms to be included in risk assessments (Table 9.1). An example of such a criterion is that the selected non-target species are of key importance in ecosystem functioning, such as natural enemies of herbivores, pollinators and decomposers. Other criteria indicate that the selected species are not only exposed to, but are also susceptible to the transgenic product, which is dependent on the expression of the gene in the plant, the specificity of the transgenic product and the feeding mechanism of the organism.

9.4.2 Evaluation of below- and aboveground systems

Plants function as essential links between aboveground and belowground organisms (van der Putten et al. 2001; Wardle et al. 2004b; Bezemer et al. 2005; Bezemer and van Dam 2005). Therefore, even if the transgene is expressed in specific tissues only, it is advised that the effects of the transgenic crop are tested for both aboveground and belowground organisms (Bruinsma et al. 2003).

9.4.3 Selection of non-target traits

The fitness of an organism is the most important non-target trait to quantify. Several performance traits, such as mortality, development time and adult weight can be used as proxies for the fitness of an organism (Roitberg et al. 2001). Changes in the values of these traits can have large effects on the populations of non-target organisms. However, many studies on non-target effects of transgenic plants only assessed mortality of the organisms. Sub-lethal effects, such as a change in development time or the sex-ratio of the population are often ignored. This can lead to an underestimation of the effect of the transgenic crop on the non-target organism (Charleston and Dicke 2008; EFSA 2010). Therefore, it is important to study lethal as well as sub-lethal effects on non-target organisms. The intrinsic rate of population growth, for example, is considered to be a good and realistic parameter for non-target studies, because it combines both lethal and sub-lethal effects (Charleston and Dicke 2008).

Effects on non-target organisms are preferably studied during multiple generations, for instance to unravel the longer term impact of sub-lethal effects (EFSA 2010). For non-target effects at the population level, long-lasting field experiments are required. For multivoltine species, which have two or more generations per year, a field experiment that is performed over an entire field season is required, and results are preferably validated during a second field season. For univoltine species, which have one generation per year, more than one field season is always required. Alternatively, modelling studies could play a role in predicting the effects of a change in the development time or fecundity on the population dynamics of non-target organisms.

Although most non-target studies focus on effects on the fitness of the non-target organisms in confined, no-choice situations, non-target testing should preferably also include effects on the selection behaviour of the organisms (Poppy and Sutherland 2004; Jongsma et al. 2010). For instance, the organisms might prefer to forage on the isogenic line or a conventionally bred variety over the transgenic variety. Such behavioural factors might limit negative effects of transgenic plants under field conditions, and ignoring effects on behaviour of non-target organisms may lead to a biased view of the potential environmental effects of transgenic plants.

Table 9.1 Criteria for selecting non-target organisms in risk assessments of transgenic crops

Criterion	Explanation	References
Ecological importance	Importance of organism in ecosystem functioning, *e.g.* pollinators, natural enemies of pest species and decomposers	(Bruinsma et al. 2003; Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Andow and Zwahlen 2006; Birch et al. 2007; Prasifka et al. 2008; EFSA 2010)
Economic importance	Importance of organism in agriculture, *e.g.* honeybees and pollinators of fruit and seed crops	(Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Prasifka et al. 2008; EFSA 2010)
Potential exposure to transgenic product	Depending on expression of the gene in the plant, on feeding behaviour of the organisms, and in the case of carnivores on abundance and feeding behaviour of their prey/host	(Bruinsma et al. 2003; Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Prasifka et al. 2008; EFSA 2010)
Susceptibility to transgenic product	Depending on specificity of the transgenic product	(Bruinsma et al. 2003; Dutton et al. 2003; Scholte and Dicke 2005; EFSA 2010)
Functional group	Selected organisms should be representatives of different functional groups, *e.g.* herbivores, pollinators, predators, parasitoids and decomposers	(Bruinsma et al. 2003; Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Andow and Zwahlen 2006; EFSA 2010)
Cultural status	Based on protected status of organism (*e.g.* endangered species) and cultural or symbolic value for society	(Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Andow and Zwahlen 2006; Prasifka et al. 2008; EFSA 2010)
Abundance in ecosystem	Selected organisms should occur in the agro-ecosystem, and abundant species are considered more likely to be important in the system	(Dutton et al. 2003; Andow and Hilbeck 2004; Scholte and Dicke 2005; Charleston and Dicke 2008; EFSA 2010)
Availability of knowledge	Information on the organism is necessary in the risk assessment study, *e.g.* for assessing ecological importance.	(Dutton et al. 2003; Scholte and Dicke 2005)
Availability and amenability	Selected organism should be commercially available or easy to keep and rear, and amendable for use in laboratory, greenhouse and field tests.	(Bruinsma et al. 2003; Dutton et al. 2003; Scholte and Dicke 2005; Birch et al. 2007; Prasifka et al. 2008; EFSA 2010)

9.4.4 Use of untargeted metabolomics

Untargeted metabolomics can be a useful tool to study the (unintended and unexpected) effects of the genetic modification on the plant's chemical properties. In this way, it can be assessed whether the metabolic profile of the transgenic crop falls outside the metabolic baseline variation, represented as described above. However, metabolomics cannot replace actual testing of these non-target effects, which is always an essential component of the ERA. In previous studies, shoot GLS profiles of several white cabbage cultivars were not comparable between greenhouse and field studies (Kabouw et al. 2010a), but nevertheless a similar cultivar ranking in insect performance/abundance for both greenhouse and field experiments was observed (Kabouw et al. 2011; Kos et al. 2011a; 2011b). This underlines the value of non-target studies.

9.5 Did the applicant interpret the results correctly?

9.5.1 Hypothesis testing

After completion of the non-target studies, it is important that the results are interpreted correctly. Statistical analysis is the final decision-making tool to assess non-target effects of the transgenic crop. In statistical analyses, a null hypothesis (set prior to the experiment) is evaluated. The null hypothesis for testing the effects of a transgenic crop on non-target organisms can be: 'the effects of the transgenic crop are not significantly different from the full complement of effects found in the baseline variation'. It is important to understand that a statistical analysis of a data set can only reject a null hypothesis or fail to reject it. If the comparison of the baseline variation and the transgenic plant reveals no statistically significant difference between them, the null hypothesis should not be rejected. Nevertheless, it does not mean that there is no difference in reality. It only means that there is not enough evidence to reject the null hypothesis.

When the null hypothesis is rejected when it should not be, a type I error occurs. This comprises a risk for the producer of the transgenic crop, because the producer has to develop a new crop in which no negative effects on non-target organisms are observed before permission for introduction in the agro-ecosystem will be granted. When the null hypothesis is not rejected when it should be, a type II error occurs. This is a risk for the environment, because the transgenic crop causes non-target effects that were not detected. The statistical power of an experiment refers to the ability to prevent type I and II errors. By increasing the sample size of an experiment, the statistical power

can be enhanced. It is important that pseudoreplication is prevented. For example, if the replicate individuals are tested as one group (*e.g.* they are in the same cage or container and get exactly the same nutrient solutions) they are not independent replicates, but together form only one replicate (see Charleston and Dicke 2008 for more information on statistical power and pseudoreplication).

9.5.2 Use of multivariate statistics (MVS) to assess non-target effects

When only one non-target trait is selected, a univariate analysis such as ANOVA (or the non-parametrical Kruskal-Wallis test) with a post-hoc multiple comparison test can be used to test whether effects of the transgenic crop differ from the baseline variation. However, experiments that address the non-target effects of transgenic crops can result in large datasets involving several traits. This large dataset might be difficult to analyse or interpret. Multivariate statistics (MVS) based on ordinations is helpful to analyse and visualise differences in suites of traits and to assess whether the genetic modification of a plant results in effects that are within or outside the baseline variation. In the context of representation of the baseline variation, MVS can also be used to identify varieties that represent extremes or averages for a set of measured traits. In general, MVS is a useful tool to classify samples and to visualise differences and relationships between samples for which multiple variables have been recorded. Principal Component Analysis (PCA), for example, converts a set of observations (variables) into a set of uncorrelated variables, called principal components. The first principal component explains the highest percentage of the total observed variation in variables, followed by the second principal component, etc. The resulting PCA plot shows how the observations are related and if there are any deviating observations in the data. In further steps of the MVS, the differences between the groups of observations (*e.g.* genotypes or environments) can be statistically analysed. In this way, it can be analysed whether the effects of the transgenic crop are different from the effects found in the baseline variation. MVS was for example used to analyse the large datasets of untargeted metabolomics of several *A. thaliana ecotypes* and to interpret the meaning of these multivariate data (Houshyani et al. 2012).

Although MVS is a useful tool for ecologists to evaluate non-target effects of transgenic crops, there is a number of challenges when using MVS. In particular, it is important to not ignore important variation and to correctly assess whether observed differences are statistically significant. Publications

using MVS frequently show and interpret only the first two principle components. However, third and higher components of ordination diagrams might still contain relevant information. On the other hand, analysis of too many principle components will result in analysing meaningless information (*i.e.* the random error). To determine the correct number of components to include, it is necessary to have an objective criterion for deciding how many components to retain for correct interpretation. There are several statistical methods to evaluate how many components should be included in MVS (see *e.g.* Zwick and Velicer 1986; Lautenschlager 1989; Watkins 2000; Hayton et al. 2004).

The second challenge is to determine whether the observed differences, *e.g.* between a transgenic plant and the baseline variation, are statistically significant. Frequently, this is tested by using the scores of the samples (*i.e.* the position of the sample in the ordination space) in more "traditional" (univariate) statistics such as ANOVA after an ordination. An alternative is to use Monte Carlo permutation tests to determine if the overall differences between groups of samples (*e.g.* plant varieties) in an MVS are statistically significant. The advantage of the Monte Carlo permutation test is that it is performed on the raw data underlying the ordination diagram and does not transform the data. This can result in a more accurate analysis of the underlying trends (Anderson and Legendre 1999; Legendre and Anderson 1999).

9.5.3 If non-target effects differ from the effects found in the baseline variation

If the non-target effects of the transgenic crop differ from the effects found in the baseline variation (the null hypothesis is rejected), or if the non-target study was not performed properly (see stepwise question list in the Supporting Information 9.1), this indicates that cultivating the transgenic crop could pose an ecological risk for non-target organisms.

9.6 Conclusions

We advise regulators to include several novel aspects in the assessment of non-target effects of transgenic crops. These are:

1) Compare non-target effects of transgenic crops with the baseline variation. Instead of only comparing the non-target effects of the transgenic crop with those of its genetic background, it is recommended

to compare the non-target effects of a transgenic crop with the baseline variation. The baseline information is necessary in order to assess whether the transgenic plant is disproportionally affecting non-target organisms compared with varieties that were produced by traditional breeding and/or were grown under a range of common environmental conditions.

2) Select several plant varieties and environments for representing the baseline variation. The baseline variation can be represented by selecting a range of genotypes/varieties with a close taxonomic relationship at the species level with the transgenic plant, and by including several appropriate environmental conditions, *e.g.* different soil types and climates.

3) Use metabolomics data for baseline representation and/or as a part of the non-target testing. Untargeted metabolomics, in which the entire metabolome of a plant is studied, is a useful tool to select the genotypes/varieties and environmental conditions to be included in the set used for representation of the baseline variation, as well as to study the effect of genetic modification on the plant's chemical properties.

4) Use multivariate statistical approaches for baseline representation and for assessment of non-target effects. Multivariate statistics (MVS) based on ordinations can be used to analyse and visualise differences in a suite of traits, which can be valuable for baseline representation and for comparing the effects of a transgenic crop with the baseline variation.


Photo: Tibor Bakonyszky

10

General Discussion

Martine Kos

10.1 Basic ecology sets the scene

10.1.1 Multitrophic effects of plant resistance

Plants can greatly influence the ecology and evolution of higher trophic levels, and they are important drivers of community structure and dynamics (Whitham et al. 2003; Crutsinger et al. 2006; Johnson et al. 2006; Bukovinszky et al. 2008; Newton et al. 2009b; Poelman et al. 2009b). Plant traits that confer resistance to herbivores can affect the natural enemies of these herbivores as well, and may even affect organisms at the fourth trophic level (Hunter and Price 1992; Harvey et al. 2003; Soler et al. 2005; Bukovinszky et al. 2008). Information on the multitrophic effects of plant resistance traits is, therefore, essential in examining the ecological effects of plants that are genetically engineered for an enhanced direct or indirect resistance to pest herbivores. In this thesis, the influence of direct and indirect plant resistance traits on the performance, behaviour and abundance of aboveground herbivores and their natural enemies was studied. This knowledge was subsequently used to address the ecological effects of plants that are genetically engineered for an enhanced direct or indirect resistance to pest herbivores.

This thesis focussed on plants of the family Brassicaceae, also known as crucifers. Among the compounds that are involved in direct and indirect plant resistance in Brassicaceae are characteristic secondary metabolites named glucosinolates (GLS) that confer direct resistance to herbivores. Upon tissue damage by herbivores, the GLS are hydrolysed by the enzyme myrosinase into several toxic compounds such as (iso)thiocyanates and nitriles that negatively affect generalist herbivores (Bones and Rossiter 2006; Halkier and Gershenzon 2006; Hopkins et al. 2009). Based on their biosynthetic origin, GLS can be categorised into three major groups, indole, aliphatic and aromatic GLS, constituting respectively 10%, 50% and 10% of the known GLS structures (Hopkins et al. 2009). Because aromatic GLS were hardly found in the foliar tissue and the phloem of the selected brassicaceous species, the results presented in this thesis focus on indole and aliphatic GLS. Indole and aliphatic GLS have different chemical and biological characteristics. Indole GLS are less stable than aliphatic GLS and some indole GLS are hydrolysed into toxic products independently of myrosinase activity (Kim and Jander 2007; Kim et al. 2008). Furthermore, indole GLS are generally more strongly induced by herbivory than aliphatic GLS (Mewis et al. 2006; Gols et al. 2008b; Textor and Gershenzon 2009). It is generally assumed that only the breakdown of aliphatic GLS yields volatile products (Hopkins et al. 2009).

Brassicaceous plants also emit other herbivore-induced volatile organic compounds (VOCs), such as terpenoids and green leaf volatiles, that attract the natural enemies of herbivores (Mattiacci et al. 1994; van Poecke and Dicke 2004; Bukovinszky et al. 2005; Mumm and Dicke 2010; Shiojiri et al. 2010). VOCs can be reliable cues indicating the presence of suitable hosts or prey for the natural enemies of herbivores feeding on brassicaceous plants (Vet and Dicke 1992; Dicke 1999a; Takabayashi et al. 2006).

The direct and indirect resistance traits of brassicaceous plants are not necessarily independent. For example, direct plant resistance traits such as the production of GLS can also affect the performance of predators and parasitoids that feed on herbivores of brassicaceous plants (Francis et al. 2001b; Vanhaelen et al. 2002; Sznajder and Harvey 2003; Soler et al. 2005). Furthermore, specialist parasitoids of herbivores that feed on GLS-containing plants are attracted to the volatile breakdown products of GLS (Bradburne and Mithen 2000; Blande et al. 2007; Mumm et al. 2008). To unravel the multitrophic effects of direct and indirect plant resistance traits in brassicaceous plants, two fundamental research objectives have been formulated **(Chapter 1).** These objectives will be discussed here.

10.1.2 Plant resistance and herbivores

To study the effects of direct and indirect plant resistance traits on herbivores, herbivores with different feeding strategies (phloem-feeding or leaf-chewing) and/or levels of specialisation (generalist or specialist), were selected, because these herbivores are probably differentially affected by plant resistance traits. The effects of plant resistance on herbivore performance and behaviour were studied in the greenhouse and the laboratory, and in the field the effects on herbivore population dynamics were examined.

10.1.2.1 Direct resistance

Under laboratory conditions, the correlations between plant GLS and performance of leaf-chewing and phloem-feeding herbivores were studied by using several ecotypes of the model plant *Arabidopsis thaliana* (L.) Heynh., which differed considerably in their GLS concentrations and profiles. It has to be noted that the ecotypes probably also differed in other chemical or morphological traits, and effects of these other differences on the performance of the herbivores cannot be ruled out. Furthermore, a transgenic line was created that produced higher concentrations of aliphatic GLS compared to the

wild-type plant. This allowed for a direct assessment of the effects of aliphatic GLS concentrations on insect performance to be made. As expected, the performance of the generalist leaf-chewing herbivore *Spodoptera exigua* Hübner (beet armyworm) correlated negatively with foliar concentrations of GLS (**Chapter 7**). This is in agreement with other studies that show a negative effect of GLS on generalist leaf-chewing herbivores (Ulmer et al. 2001; Gigolashvili et al. 2007; Arany et al. 2008; Beekwilder et al. 2008; Müller et al. 2010). Generalist herbivores are usually less adapted to feed on plants containing specific secondary metabolites than specialist herbivores (van der Meijden 1996; Schoonhoven et al. 2005; Berenbaum and Zangerl 2008; Gols and Harvey 2009). Specialist herbivores of Brassicaceae have evolved specific adaptations to detoxify GLS or inhibit the formation of toxic (iso) thiocyanates (Ratzka et al. 2002; Wittstock et al. 2004), or may sequester GLS for their own benefit (Francis et al. 2001b; Kazana et al. 2007; Müller 2009). Specialist herbivores can also use GLS and their hydrolysis products as oviposition or feeding stimulants (van Loon et al. 1992; Gabrys and Tjallingii 2002; Miles et al. 2005). The small cabbage white butterfly *Pieris rapae* L., a leaf-chewer specialised on brassicaceous plants, possesses a nitrile-specifier protein (NSP) in the gut that diverts GLS degradation from toxic isothiocyanates to less toxic nitriles (Wittstock et al. 2004). However, in contrast to other studies (Gols et al. 2008b; Müller et al. 2010), a negative correlation between GLS concentrations and the performance of *P. rapae* was found (**Chapter 7**). This finding suggests that *P. rapae* cannot completely prevent the formation of toxic GLS hydrolysis products, probably because the NSP is not equally efficient in inhibiting the breakdown all GLS compounds into toxic products (Gols et al. 2008b; H. Vogel personal communication). This hypothesis is supported by the excretion of isothiocyanates in the faeces of *P. rapae* (Agelopoulos et al. 1995), indicating that isothiocyanates are indeed formed in the gut of this species. Perhaps brassicaceous plants have evolved ways to circumvent the detoxification or diversion mechanism of specialist herbivores by increasing the production of specific GLS compounds that these herbivores cannot detoxify. Plants may maintain a large variation in their chemical profile to confer resistance to many different herbivores (Jones and Firn 1991; Newton et al. 2009b; Poelman et al. 2009b).

Phloem-feeding herbivores such as aphids have a different feeding strategy than leaf-chewing insects. Due to the intercellular path taken by the aphid stylet to the phloem (Tjallingii and Hogen Esch 1993), aphids cause

relatively little tissue damage, and different signalling pathways are induced, compared to feeding by leaf-chewing herbivores (de Vos et al. 2007; de Vos and Jander 2010). Aphids can ingest GLS from the phloem without bringing these compounds into contact with plant myrosinases that are stored in cells adjacent to the phloem (Andreasson et al. 2001). Thus, aphids can prevent the breakdown of GLS by plant myrosinases (de Vos et al. 2007; Kim and Jander 2007). The cabbage aphid *Brevicoryne brassicae* L. is a specialist phloem-feeding herbivore that uses GLS as feeding stimulants (Gabrys and Tjallingii 2002), and therefore it was expected that GLS would positively influence its performance. As expected, the performance of this aphid was positively correlated with concentrations of aliphatic GLS in the phloem of its host plant (**Chapter 6**). Interestingly, also a positive correlation between indole GLS and aphid performance was observed (**Chapter 6**, also observed in **Chapters 4 and 5** with white cabbage). This was unexpected, because indole GLS have been shown to be hydrolysed in aphids into toxic products independently of myrosinase activity (Kim and Jander 2007; Kim et al. 2008), and negative correlations between indole GLS and *B. brassicae* performance have been reported before (Cole 1997; Mewis et al. 2005). Perhaps specific indole GLS affect aphid performance more strongly than others, and qualitative differences in indole GLS between this thesis and the literature may have caused the contrasting results.

In the field, four white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba*) cultivars were used to study the correlation between chemical and morphological resistance traits and the population dynamics of two specialist herbivores (**Chapter 4**). A number of both chemical and morphological plant traits correlated with the abundance of *B. brassicae* and the leaf-chewing lepidopteran *Plutella xylostella* L (diamondback moth). Both species displayed similar responses to the chemical and morphological plant traits, despite their difference in feeding strategy. The difference in abundance on the different white cabbage cultivars was, however, larger for *B. brassicae* than for *P. xylostella*. This is probably due to the rapid, exponential population growth of *B. brassicae* that allows for a faster increase in abundance on the most suitable host plant (Broekgaarden et al. 2008). A positive correlation was found between abundance of the herbivores and concentrations of most aliphatic and indole GLS, but a negative correlation was found for the aliphatic glucoiberin. These correlations were consistent over two study years. The cultivars did not differ consistently in concentrations of total, indole or aliphatic

GLS between both study years, and the differences in the concentrations of these classes of GLS did not explain the difference in herbivore abundance. Based on the results, it was suggested that in *B. oleracea* specific GLS might be more important for herbivore abundance than total concentrations of these compounds. Leaf toughness and amount of leaf surface wax of white cabbage plants correlated negatively with abundance of the herbivores, suggesting that plants employ both chemical and morphological resistance traits to defend themselves against herbivores.

The results presented in this thesis indicate that there is a considerable intraspecific variation in resistance to herbivores both a) between cultivars of a crop species, and b) between plant ecotypes of a wild species. This intraspecific variation led to large differences in performance and abundance of aboveground herbivores. Intraspecific plant variation also affected abundance of root-feeding herbivores in our system (Kabouw et al. 2010b). The results are in agreement with other studies that show that intraspecific variation in plant resistance traits can have large effects on the herbivore community (Whitham et al. 2003; Johnson and Agrawal 2005; Bailey et al. 2006; Crutsinger et al. 2006; Newton et al. 2009b; Poelman et al. 2009b).

10.1.2.2 Indirect resistance

VOCs are not only used by natural enemies of herbivores to find their host or prey, they also play a role in the interactions with other community members, such as herbivores, pathogens, pollinators, and neighbouring plants (Dicke and van Loon 2000; Bruinsma and Dicke 2008; Dicke and Baldwin 2010; Kessler and Heil 2011; Lucas-Barbosa et al. 2011). The effect of enhanced indirect plant resistance on the performance and behaviour of the aphid *B. brassicae* was studied by using transgenic *A. thaliana* plants that emitted one novel terpenoid and increased amounts of two endogenous terpenoids, compared to wild-type plants (**Chapter 8**). The modified emission of the terpenoids did not affect aphid performance, in agreement with a similar study using a leaf-chewing herbivore (Yang 2008), suggesting that these terpenoids are probably not directly toxic to herbivores at the levels expressed. The enhanced emission of the terpenoids did, however, affect the behaviour of *B. brassicae*: the transgenic plants repelled the aphids. There is a large body of literature on the role of herbivore-induced VOCs as repellents to (mostly generalist) herbivores, presumably by indicating that 1) the plant has initiated its resistance, 2) potential competitors are present on the plant, and/or 3) the

plant might be attractive to natural enemies (see *e.g.* Dicke 1986; de Moraes et al. 2001; Kessler and Baldwin 2001; Horiuchi et al. 2003; Heil 2004; Sanchez-Hernandez et al. 2006; Yang 2008). In contrast, several other studies demonstrate the role of herbivore-induced VOCs as attractants for (mostly specialist) herbivores, probably by indicating the presence of a suitable host plant (see *e.g.* Bolter et al. 1997; Kalberer et al. 2001; Heil 2004; Carroll et al. 2006; Halitschke et al. 2008).

10.1.2.3 Relative importance of bottom-up and top-down forces

In the field, it was assessed whether herbivore abundance is more strongly affected by bottom-up forces (plant chemistry and morphology) than by plant-mediated top-down forces (attraction of natural enemies) (**Chapter 4**). As a measurement of natural enemy attraction, parasitism of herbivores and predator oviposition on the four white cabbage cultivars was quantified. The observed differences in parasitism and oviposition among the cultivars did not explain the observed differences in herbivore abundance among the cultivars. This suggests that in this study system plant chemistry and morphology (bottom-up) were more important for herbivore abundance than attraction and arrestment of natural enemies (plant-mediated top-down). The results of this thesis are in line with the general view that in terrestrial systems bottom-up forces are considered to be stronger than top-down forces (Hunter and Price 1992; Schmitz et al. 2000; Halaj and Wise 2001; Walker et al. 2008). This may be partly due to the potential for compensation of a decrease in the herbivore population by an increase in plant consumption by the remaining herbivores, and the complexity and high species diversity of terrestrial ecosystems that reduce the effect of a single carnivore species (Schmitz et al. 2000).

10.1.3 Plant resistance and carnivores

10.1.3.1 Direct resistance

Effects of secondary plant metabolites are not confined to the second trophic level. Plant metabolites can also negatively influence the performance of carnivores that feed on herbivores containing these metabolites. This can be a direct effect due to toxicity of the metabolites, or an indirect effect due to reduced growth and development of the host or prey (Harvey 2005; Soler et al. 2005; Ode 2006; Gols and Harvey 2009). In a large-scale field experiment, it was indeed observed that plant resistance traits correlate with the abundance of the predators and parasitoids in the field (**Chapter 4**). In greenhouse and

laboratory experiments, the mechanisms underlying the herbivore-mediated effects of secondary metabolites on natural enemies were addressed. Natural enemies belonging to different functional groups, *i.e.* parasitoids and predators, were selected for this purpose. Whereas predators kill their prey immediately and feed on multiple prey items during their development, koinobiont parasitoids develop inside a single host individual and feed selectively on certain host tissues (Godfray 1994). Therefore, it was expected that these natural enemies are differentially affected by herbivore-mediated plant resistance traits. Effects of GLS on natural enemies depend not only on the feeding strategy of the natural enemy, but also on the biology of the herbivore (Gols and Harvey 2009). For example, some specialist herbivores have evolved specific adaptations to detoxify or excrete GLS (Francis et al. 2001b; Ratzka et al. 2002; Wittstock et al. 2004), and effects of GLS might therefore be less pronounced in the natural enemy than in the herbivore (Gols et al. 2008b). The performance of one of the parasitoids of *P. rapae*, *Hyposoter ebeninus* Gravenhorst, was studied using wild-type and transformed *A. thaliana* lines with, among others, considerable differences in GLS profiles (**Chapter 7**). As discussed in paragraph 10.1.2, *P. rapae* performance was negatively correlated with GLS in the host plant. It was expected that, through a decreased host quality on the high-GLS containing plants, the performance of the parasitoid would also negatively correlate with plant GLS. Surprisingly, the percentage of successful parasitism by *H. ebeninus* was highest when its host developed on the high-GLS containing plants, possibly caused by negative effects on host immune responses imposed by biochemical processes inhibiting the formation of toxic GLS-breakdown products that take place in the host. This indicates that GLS are not only implied in direct resistance to herbivores by negatively affecting their performance, but also in indirect resistance by increasing the performance of the parasitoids of these herbivores. These results suggest that in certain tritrophic systems, GLS can have a dual defensive role.

Some specialist herbivores can take advantage of the plant's resistance mechanism by sequestering the secondary metabolites from the plant and using these for their own defence against their attackers (Duffey 1980; Müller 2009). For example, *B. brassicae* sequesters GLS from its food plant into the haemolymph (Francis et al. 2001b; Kazana et al. 2007; Pratt 2008). The aphid contains an endogenous myrosinase that is stored separately from the GLS in the non-flight muscles (Jones et al. 2001; Bridges

et al. 2002; Francis et al. 2002). *Brevicoryne brassicae* selectively sequestered GLS from the phloem (**Chapters 3, 5 and 6**). The aphids contained mainly aliphatic GLS. Aliphatic GLS are degraded more by purified aphid myrosinase, whereas the lowest activities of the aphid myrosinase are observed with indole GLS (Francis et al. 2002), and higher sequestration of aliphatic GLS by *B. brassicae* may therefore lead to higher toxicity to predators upon damaging the aphid, without affecting aphid performance itself. Larvae of the predators *Episyrphus balteatus* de Geer and *Chrysoperla carnea* Stephens kill their prey by sucking out the fluids and they feed on multiple prey individuals during their development. The performance of *E. balteatus* and *C. carnea* was negatively affected by the sequestration of GLS by *B. brassicae* (**Chapters 3 and 6**). It has been reported before that predators of *B. brassicae* are negatively affected by high GLS concentrations in the aphid (Francis et al. 2001b; Vanhaelen et al. 2002; Kazana et al. 2007; Pratt 2008; Chaplin-Kramer et al. 2011). Effects on aphid parasitoids, however, have been rarely studied. The parasitoid *Diaeretiella rapae* McIntosh develops inside a single aphid individual. Its larvae consume host haemolymph during most of their larval development, thereby preventing damage of the tissue in which the myrosinase is stored, and only consume other host tissues at the end of the development (Godfray 1994; Harvey et al. 2000). Thus, the larvae can possibly prevent the breakdown of GLS into toxic products during the major part of their development. Indeed, the performance of this parasitoid seemed not affected by GLS concentrations in its host (**Chapter 6**). Parasitoid performance seemed most affected by the performance (size) of the host itself (**Chapters 5 and 6**), in agreement with other studies using different parasitoid species (Harvey 2005; Bukovinszky et al. 2008). These results suggest that natural enemies with a different feeding strategy, *i.e.* predators and parasitoids, are differentially affected by the sequestered GLS in *B. brassicae*.

Morphological plant traits, such as epicuticular wax or trichomes, potentially affect carnivores foraging on the plant (Eigenbrode 2004; Verheggen et al. 2009). In this thesis, there were indications of a negative effect of the trichome density of *A. thaliana* on the performance and behaviour of *E. balteatus*, although the experiments were not designed to test this (**Chapter 6**). However, no negative effect of trichome density on the behaviour of *E. balteatus* was observed in a different experiment (**Chapter 8**). Hence, no definitive conclusions about the effects of plant morphology on the natural enemies studied can be drawn from the studies presented in this thesis.

Similar to what was discussed for herbivores (paragraph 10.1.2), the results discussed above indicate that there is a large effect of intraspecific variation in direct resistance traits in both cultivated and wild plant species on the performance and abundance of natural enemies of herbivores. It is known that plant resistance traits can determine species diversity and population dynamics at higher trophic levels (Hunter and Price 1992; Bukovinszky et al. 2008), and that intraspecific plant variation can have large effects on the entire insect community (Crutsinger et al. 2006; Johnson et al. 2006; Newton et al. 2009b; Poelman et al. 2009b).

10.1.3.2 Indirect resistance

The herbivore-induced emission of plant VOCs results in attraction of natural enemies of herbivores (Turlings et al. 1990; Vet and Dicke 1992; Takabayashi et al. 1994; Karban and Baldwin 1997; Dicke and Baldwin 2010; Shiojiri et al. 2010; Kessler and Heil 2011). In this thesis, the attraction of natural enemies towards VOCs was tested only in the laboratory, but Poelman et al. (2009a) showed that preference of parasitoids in the laboratory can reliably predict their parasitism rates in the field. Different species of natural enemies may respond differently to the emission of VOCs, depending *e.g.* on their host or prey range. My results showed that aphid-infested plants of three *A. thaliana* ecotypes differed in the VOC blend emitted. The *A. thaliana* ecotype that emitted the largest amounts of volatile GLS breakdown products was most attractive to the aphid parasitoid *D. rapae* (**Chapter 6**), confirming that direct and indirect plant resistance are not necessarily mutually exclusive (Reudler Talsma 2007; Gols and Harvey 2009). It was found previously that *D. rapae*, which has a broad host range (Pike et al. 1999) but is the main parasitoid of several *Brassica*-feeding aphids (Bukovinszky et al. 2008), is attracted to plants emitting volatile breakdown products of GLS (Read et al. 1970; Bradburne and Mithen 2000; Blande et al. 2007). In contrast to *D. rapae*, adults of the aphid predator *E. balteatus* were repelled by the *A. thaliana* ecotype that emitted larger amounts of volatile GLS breakdown products. This ecotype supported a lower performance of the larvae of this predator when fed with aphids reared on this ecotype. Both the parasitoid and the predator preferred the ecotype on which their offspring performed best, confirming that preference and performance of carnivores are often positively correlated (Soler et al. 2007; Gols et al. 2009).

The parasitoid *D. rapae* preferred transgenic, aphid-infested *A.*

thaliana plants with a novel or increased emission of three terpenoids that are commonly induced by herbivore feeding over aphid-infested wild-type plants (**Chapter 8**). The broad host range of *D. rapae* may explain why this parasitoid was attracted towards plants emitting larger amounts of these common herbivore-induced volatiles. The generalist predator *E. balteatus* did not differentiate between transgenic or wild-type plants, probably because the predator has a low olfactory sensitivity for these terpenoids, as was shown by electroantennographic (EAG) studies by Verheggen et al. (2008).

In the field, there were no consistent differences in the rate of parasitism by *D. rapae* and *Diadegma semiclausum* Hellén and the rate of oviposition by the predators *E. balteatus* and *C. carnea* between aphid-infested plants of four white cabbage cultivars (**Chapter 4**). Because it has been observed that field parasitism rates of parasitoids correspond to the preference behaviour of these parasitoids in the laboratory (Poelman et al. 2009a), my results suggest that the cultivars did not differentially affect the behaviour of the tested parasitoids and predators.

In agreement with previous studies (Agrawal et al. 2002; Hoballah et al. 2002; Rasmann et al. 2005; D'Alessandro et al. 2006; Shiojiri et al. 2006; Gols et al. 2009; Poelman et al. 2009a; Snoeren et al. 2010), the results presented in this thesis indicate that intraspecific differences in the quality or quantity of volatile blends, either natural or obtained through genetic engineering, can result in a differential attraction of natural enemies. This might lead to plant-mediated differences in the top-down control of herbivores in the field.

10.1.4 Conclusion fundamental objectives

The results presented in this thesis show that plant resistance traits differentially affect the performance of herbivores with different feeding strategies, *i.e.* phloem-feeders and leaf-chewers. Through direct or herbivore-mediated effects, plant resistance traits can also differentially affect the performance and behaviour of natural enemies with different feeding strategies, *i.e.* predators and parasitoids. The results suggest that direct and indirect resistance traits can be in conflict, but they can also work in concert to enhance resistance to herbivores, depending on the biology of the herbivorous and carnivorous species involved (Fig. 10.1). Through the different direct and indirect effects, plant resistance traits affect insects at multiple trophic levels, and genetic variation in these resistance traits can have large effects on the

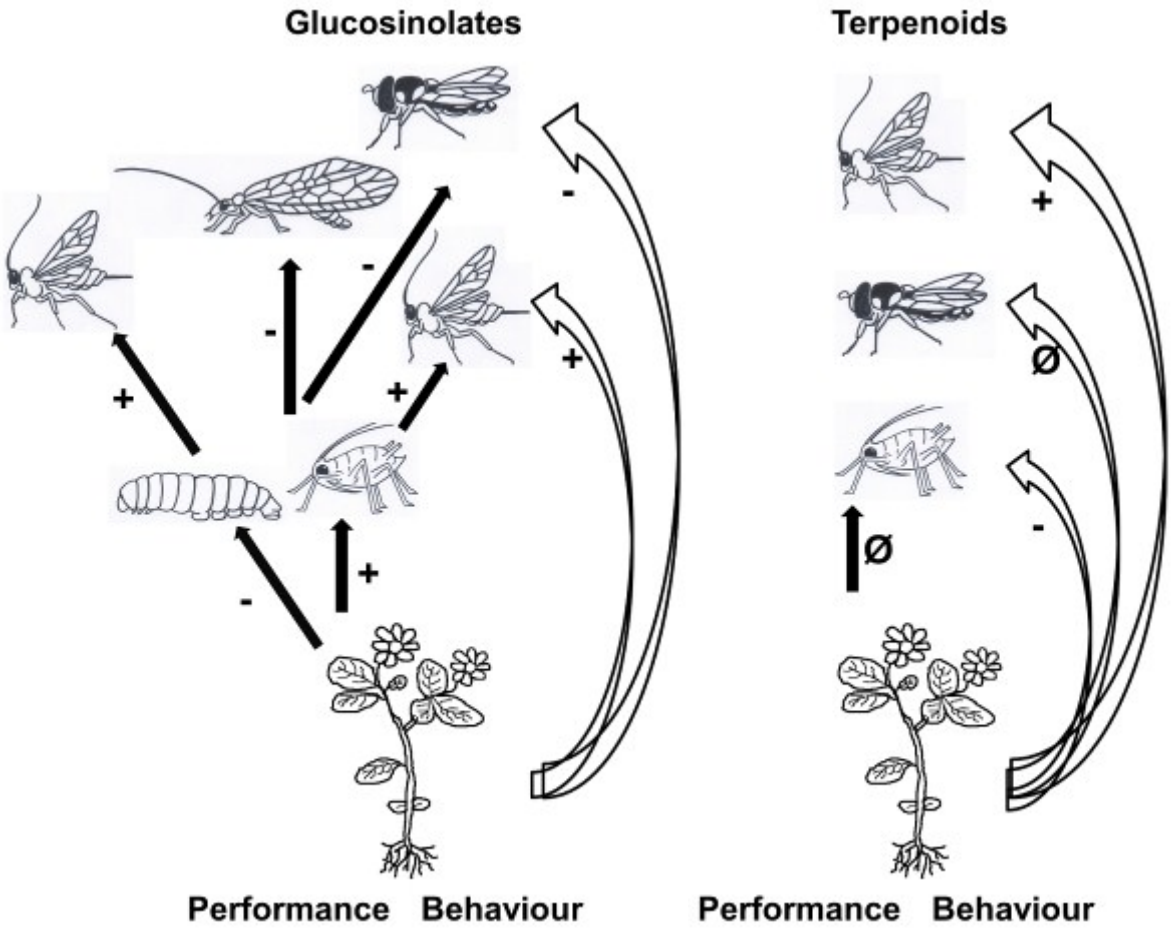


Fig. 10.1. *The correlations between plant glucosinolates (left) or terpenoids (right) and the performance (filled arrows) or behaviour (open arrows) of herbivores and carnivores with different feeding strategies under controlled greenhouse and laboratory conditions, as reported in this thesis. + refers to a positive correlation, - refers to a negative correlation, and Ø refers to no correlation. Results were obtained by using several B. oleracea cultivars and wild-type and genetically transformed Arabidopsis thaliana lines. Glucosinolates (GLS): Concentrations of GLS in the plant correlated negatively with performance of the specialist lepidopteran Pieris rapae, but positively with the performance of its parasitoid Hyposoter ebeninus, possibly caused by negative effects on host immune responses. Performance of the specialist aphid Brevicoryne brassicae correlated positively with concentrations of plant GLS. The aphid selectively sequestered GLS from the host plant and stored these in its body. Concentrations of sequestered GLS in the aphid correlated negatively with the performance of two of its predators, the hoverfly Episyrphus balteatus and the lacewing Chrysoperla carnea, but positively with that of one of its parasitoids, Diaeretiella rapae. The emission of larger amounts of volatile breakdown products of plant GLS correlated with a higher attractiveness to D. rapae, but repellence to E. balteatus. Terpenoids: The emission of larger amounts of volatile terpenoids by transgenic A. thaliana plants repelled the aphid B. brassicae, but did not affect its performance. The emission of larger amounts of terpenoids attracted D. rapae, but did not affect the behaviour of E. balteatus. Insect drawings by Cindy ten Broeke.*

entire insect community (Crutsinger et al. 2006; Johnson et al. 2006; Bukovinszky et al. 2008; Newton et al. 2009b; Poelman et al. 2009b).

10.2 From basic ecology to application in transgenic crops

10.2.1 Ecological concerns of transgenic crops

The global use of transgenic plants has increased since their commercialisation in the mid 1990s (James 2010). Transgenic crops may become vital components of Integrated Pest Management (IPM) (**Chapter 2**; Romeis et al. 2008a). Because there are several ecological concerns related to the introduction of transgenic plants in agro-ecosystems, the discussion on the urgency of obtaining insight in the ecological effects of transgenic crops increased during the last decade. However, until recently, there were no guidelines available with regard to how ecological risk assessments of transgenic crops should be carried out. The acquisition of ecological knowledge on transgenic crops, which is of paramount importance to be able to assess the ecological risk of transgenic crops, lagged behind the biotechnological developments (Schuttelaar & Partners et al. 2004). Therefore, in 2007, the Dutch government issued the ERGO (Ecology Regarding Genetically modified Organisms) programme. The main objective of the ERGO -programme was to study the ecology of transgenic crops to be able to develop ecology-based guidelines for assessing the ecological effects of new transgenic crops. Of the ecological concerns related to the introduction of transgenic crops in agro-ecosystems, the potential negative effects on non-target organisms are of key importance (Groot and Dicke 2002; Dutton et al. 2003; Romeis et al. 2006; van Lenteren 2008; Gagic et al. 2011; van Lenteren 2012; **Chapter 2**). Therefore, one of the projects within the total ERGO-programme focussed specifically on developing an ecological method to evaluate the effects of transgenic crops on non-target organisms. The current thesis is one of the three theses resulting from this project and focussed, for pragmatic reasons, on one specific group of non-target organisms: the carnivorous arthropods (predators and parasitoids).

During the execution of the ERGO-programme and the writing of the current thesis, the EFSA (European Food Safety Authority) acknowledged the lack of clear guidelines for testing non-target effects of transgenic crops. In 2010, the EFSA published the ‘Guidance of the environmental risk assessment of genetically modified plants’. This document addresses seven specific areas of concern, among which the interaction of the transgenic plant with non-target

organisms. The document provides general guidelines that specify what functional groups of non-target species to include, what endpoints to measure, and how to perform experiments under laboratory conditions (EFSA 2010). However, the document does not provide much information on the development of field experiments, use of novel techniques such as 'omics analyses, what the appropriate baseline is against which the ecological significance of any non-target effects should be assessed, and how the results from the non-target study can be correctly interpreted. These aspects of testing effects of transgenic crops on non-target organisms were considered of the utmost importance in the ERGO-programme.

The basic ecological knowledge on the multitrophic interactions between brassicaceous plants and insects that was presented in paragraph 10.1 was used to address the applied research objectives that have been formulated at the start of the project (**Chapter 1**). These objectives will be discussed here.

10.2.2 Baseline variation

In this thesis, the baseline variation is defined as 'the variation in effects observed among a selection of non-transgenic varieties, across sets of environmental conditions' (**Chapters 1 and 9**). Four white cabbage cultivars, as well as three *A. thaliana* ecotypes, were selected to represent the baseline variation. Several species of parasitoids and predators were chosen as non-target organisms. Their host and prey herbivores were also included in the experiments, because effects of transgenic plants on the herbivores (the target organisms) might explain the effects on their natural enemies (the non-target organisms). The selected *B. oleracea* varieties and *A. thaliana* ecotypes differed considerably in the production of secondary metabolites, resistance to herbivores and effects on the performance and behaviour of the natural enemies of these herbivores (**Chapters 3-8**), suggesting that these varieties and ecotypes represented a sufficiently broad range of non-target effects. Large differences in the chemistry and the interaction with insects among both conventionally bred varieties of crop species (see *e.g.* Gouinguene et al. 2001; Hoballah et al. 2002; Daun 2004; Kushad et al. 2004; Bukovinszky et al. 2009; Poelman et al. 2009b) and ecotypes/populations of wild species (see *e.g.* Kliebenstein et al. 2001; Johnson and Agrawal 2005; Gols et al. 2008b; Newton et al. 2009b; Huang et al. 2010; Snoeren et al. 2010; Houshyani et al. 2012) have been reported before.

The ranking of the white cabbage cultivars for herbivore and natural enemy abundance in the field was consistent over two field seasons (**Chapter 4**). Furthermore, the cultivar ranking in the field was similar to its ranking for herbivore and natural enemy performance in two greenhouse experiments, using different soil types (**Chapters 3 and 5**). Moreover, the effect of the plant cultivar was more important for aboveground multitrophic interactions than the soil treatment (**Chapter 5**). These results suggest that, in this study system, the baseline variation in effects on target and non-target organisms is consistent over different environments, soil types and time. It has been suggested before that genotypic effects can be stronger than environmental effects in shaping insect communities (Johnson and Agrawal 2005; Bangert et al. 2006; Poelman et al. 2008c). Effects of the plant genotype can extend to the third trophic level, and even beyond (Bailey et al. 2006; Bukovinszky et al. 2008; Poelman et al. 2008c).

10.2.3 From baseline variation to transgenic effects

Genetically transformed *A. thaliana* lines with an enhanced direct and indirect resistance were produced to study their effects on the performance and behaviour of target and non-target organisms. In the experimental chapters of this thesis, the effects of the transformed line were compared only with those of the isogenic line (the untransformed wild-type line). The reason for this was that from a scientific point of view, this is the only relevant comparison to assess the effects of a single gene affecting a plant resistance trait (for example GLS concentration and VOC emission) on insects. After all, the three *A. thaliana* ecotypes that were used in the experimental studies differed in many more traits than just the resistance trait we were interested in. For example, different *A. thaliana* ecotypes have been shown to differ in production of primary (*e.g.* carbohydrates and amino acids) and secondary metabolites (*e.g.* GLS and VOCs) (Kliebenstein et al. 2001; Huang et al. 2010; Snoeren et al. 2010; Houshyani et al. 2012) and in morphological traits such as leaf size and trichome density (Passardi et al. 2007). Comparing the performance of insects on a transformed line of a certain ecotype with their performance on wild-type lines of other ecotypes is, however, relevant from an applied point of view. It is important that the effects of a transformed plant are compared with the effects observed in the baseline variation, not just with the effects of the isogenic line. Therefore, additional statistical analyses were performed that included the transformed line(s) and all wild-type lines. The

results from these additional analyses are discussed below.

For a modified direct resistance, one transformed *A. thaliana* line with enhanced foliar aliphatic GLS concentrations was produced from ecotype Col-0. The effects of this transformed line on the performance and behaviour of the selected non-target organisms were usually not different from the baseline variation in these effects, represented by the three *A. thaliana* ecotypes Col-0, Cvi and Eri (**Chapters 6 and 7**). This was probably because most of the traits of the transformed plant, such as GLS and VOC profile, biomass, and trichome density fell within the baseline variation as well (Fig. 10.2; **Chapters 6 and 7**). Because Col-0 had much lower GLS concentrations than ecotype Cvi, even overexpression of the HAG1/MYB28 transcription factor in Col-0 did not result in GLS concentrations that were higher than the concentrations naturally present in Cvi (**Chapters 6 and 7**). From ecotype Cvi, we were able to generate transgenic lines over-expressing the HAG1/MYB28 transcription factor, but none of the produced transgenic lines demonstrated an enhanced GLS production (data not shown), perhaps because this ecotype already produces very high concentrations of GLS and there are physiological limits to the level of GLS biosynthesis.

For a modified indirect resistance, a transgenic *A. thaliana* line expressing the linalool/nerolidol synthase gene *FaNES1* from strawberry was generated from each of three ecotypes (An-1, Col-0 and Eri). Apart from the expected higher emission of three terpenoids, there were no differences in the overall volatile profile between the transgenic plants and the baseline variation. The aim of the genetic engineering was to enhance the attraction of natural enemies of aphids to the transgenic plants. This was successful: a preference of a parasitoid wasp towards the transgenic plant was recorded for two of the ecotypes, although no effect on the behaviour of a predator was observed (**Chapter 8**). Interestingly, the aphid itself was repelled by the transgenic *FaNES*-expressing plants of two of the ecotypes, a phenomenon also reported for other herbivore species (Aharoni et al. 2003; Yang 2008). The performance of the aphid was not affected by the transformation, and differences in aphid performance among the ecotypes were larger than between each transgenic line and the corresponding wild-type line (**Chapter 8**). Because there was no effect of the transgenic plants on the performance of the aphid, it can be expected that, in the field, the repellence of this aphid by the transgenic plants will disappear over time.

There were some unexpected changes in the chemistry and

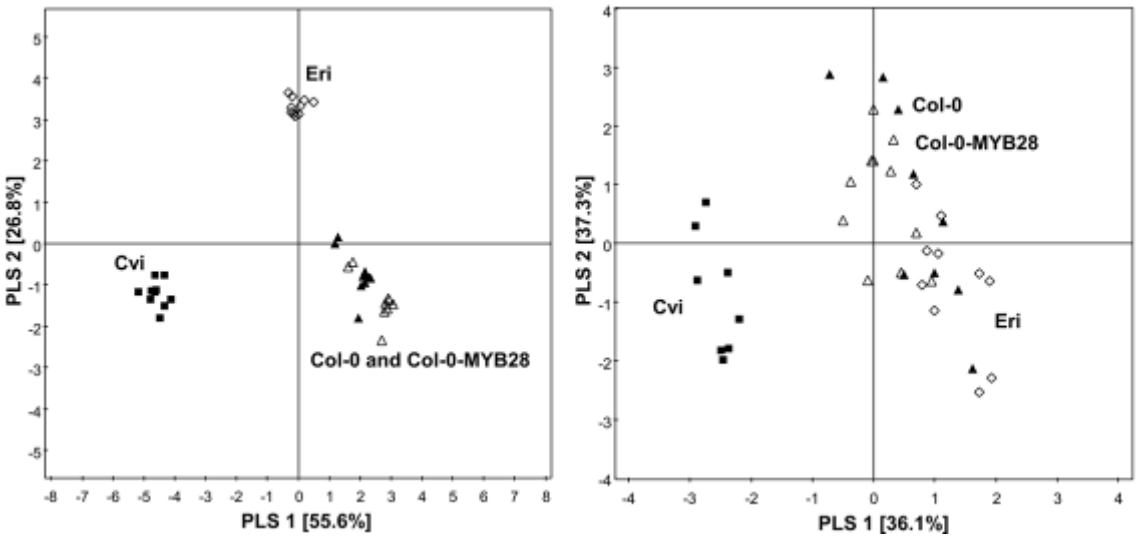


Fig. 10.2 *PLS-DA score plot showing the distinction in foliar glucosinolate profile (left) and volatile organic compound profile (right) among three Arabidopsis thaliana ecotypes [Cvi (boxes), Eri (diamonds) and Col-0 (filled triangles)] and one genetically transformed line [Col-0-MYB28 (open triangles)]. In brackets the percentage of variation explained by each axis is indicated. See* ***Chapter 6*** *for more information.*

morphology of the transformed plants. For example, Col-0-MYB28 plants overexpressing a gene involved in the aliphatic GLS pathway produced, as planned, higher concentrations of aliphatic GLS in the leaves, but unexpectedly lower concentrations of these compounds in the phloem (**Chapter 6**). Furthermore, transgenic plants with modified VOC emission differed significantly in biomass, diameter, number of leaves and trichome density with the corresponding wild-type plants (**Chapter 8**). These pleiotropic effects on plant chemistry and morphology did not seem to lead to a significant effect on the performance or behaviour of the target and non-target organisms.

Although most non-target studies focus only on effects on the fitness of the non-target organisms, non-target testing preferably also includes effects on behaviour (**Chapters 2 and 9**; Poppy and Sutherland 2004; Jongsma et al. 2010). Predators and parasitoids have the ability to select the plant on which their offspring performs best (**Chapter 6**; Soler et al. 2007; Gols et al. 2009), and behavioural factors might therefore limit potential detrimental effects of a transgenic plant on these non-target organisms. Natural enemies are capable of learning the odours that they associate with a rewarding experience, such as a positive oviposition event in a host or feeding on a prey (Lewis and

Tumlinson 1988; Vet and Dicke 1992; Vet et al. 1995; de Boer et al. 2005; D'Alessandro et al. 2006; Takabayashi et al. 2006; Allison and Hare 2009; Hoedjes et al. 2011). However, natural enemies might also learn from unrewarding experiences (Papaj et al. 1994; Vet et al. 1998). In the case of transgenic plants that constitutively emit larger amounts of VOCs, natural enemies might learn that the constitutive signal does not indicate the presence of their prey or host and might consequently ignore the signal (Degenhardt et al. 2003; Poppy and Sutherland 2004). These examples stress the importance of including behavioural studies in the ecological risk assessments of transgenic crops.

For the systems studied, the effects of the transformed line on aboveground non-target insects were usually within the baseline variation. This indicates that the transformed plants were not disproportionally affecting aboveground non-target organisms compared to the wild ecotypes. Most studies on the effects of commercially grown transgenic crops, which all express genes coding for *Bacillus thuringiensis* (*Bt*) toxins, indicate negligible effects on non-target arthropods (as reviewed in *e.g.* Groot and Dicke 2002; Dutton et al. 2003; Romeis et al. 2006; Thies and Devare 2007). From the studies discussed in these review papers it can be concluded that parasitoids and predators are generally not directly affected by *Bt* toxins. Although a reduction in the abundance of their host or prey in *Bt* fields could potentially lead to negative effects on the abundance of parasitoids and predators, these effects can be expected when using any kind of pest control, and are therefore not different from conventional agriculture in which pesticides are used (Dutton et al. 2003). A study on the arthropod community on *Bt* rice showed no difference in the diversity or the dominance distribution of several arthropod groups (phytophages, parasitoids, predators, detritivores and others) between *Bt* and non-*Bt* rice fields (Li et al. 2007). Another study on transgenic disease-resistant wheat reported that the variation in effects on aphid-parasitoid food webs between conventionally bred wheat varieties was as large as between a transgenic plant and its control, and the authors suggested that the effects of the transgenic plants on non-target insects may therefore be limited (von Burg et al. 2011). Although most studies report negligible effect of *Bt* crops on non-target organisms, there is a limited number of studies that do report significant negative effects on non-target organisms, mostly on insects of the same taxonomic order as the target herbivore. Probably the best known example is that of the monarch butterfly (*Danaus plexippus* L.). Losey et al. (1999)

published a scientific correspondence in *Nature* with the title "*Transgenic pollen harms monarch larvae*", in which possible deleterious effects of transgenic *Bt* pollen from a specific transgenic maize variety on monarch butterflies under laboratory conditions were reported. Subsequent laboratory and field studies with the same transgenic variety confirmed these results (Hansen and Obrycki 2000; Hellmich et al. 2001; Stanley-Horn et al. 2001), although some of these studies received a lot of criticism from other authors (see *e.g.* Shelton and Sears 2001). After publication of these studies, the studied transgenic maize variety was taken off the market (Groot and Dicke 2002; Thies and Devare 2007).

10.2.4 The predictive value of greenhouse experiments

One of the goals of the ERGO-project was to assess the validity of using greenhouse experiments to predict non-target effects in the field. The combined results from this thesis and the other two theses of the ERGO-project (Houshyani 2012; Kabouw 2012) show that greenhouse studies can often provide good indications for the effects that can be expected in the field (see **Chapter 9** for details). For example, for performance and abundance of aboveground herbivores and their natural enemies, a similar ranking of the cultivars for both greenhouse and field experiments was observed (**Chapters 3, 4 and 5**). However, the shoot GLS profiles of the plants these insects were developing on, were highly variable and not comparable between the greenhouse and field experiments (Kabouw et al. 2010a), indicating that results from the greenhouse cannot always reliably predict results from the field. Furthermore, the single plant-organism interactions and the homogenised potting soil that are tested in the greenhouse might not be representative for the complexity of interactions in the field. Thus, field studies are always required later in the risk assessment procedure to validate the results from the greenhouse (**Chapter 9**). In agreement with this recommendation, soon after publication of the study by Losey et al. (1999) that reported negative effects of *Bt* pollen on monarch butterflies under laboratory conditions, field studies were performed to validate the results from the laboratory under field conditions (Hansen and Obrycki 2000; Stanley-Horn et al. 2001).

10.2.5 Guidelines for assessing non-target effects

Chapter 9 presents guidelines that can be used to assess the effects of

transgenic crops on non-target organisms. These guidelines were developed in a combined effort of the three PhD-projects involved in this ERGO-programme, and concern both below- and aboveground systems. One of the novel aspects of these guidelines is to compare the non-target effects of transgenic crops with the baseline variation in these effects, in order to assess whether the transgenic plant is disproportionally affecting non-target organisms compared to the varieties that were produced by traditional breeding. We have suggested to select several plant varieties and realistic environmental conditions for a proper representation of the baseline variation (**Chapter 9**).

10.2.6 Conclusion applied objectives

In this thesis, a considerable variation in plant resistance traits and consequentially considerable variation in effects on target and non-target organisms in both cultivated and wild brassicaceous plant species was observed. The baseline variation in effects on target and non-target organisms was relatively consistent over different environments, soil types and time. The effects of transformed *A. thaliana* plants altered in direct and indirect resistance on non-target organisms were mostly not different from the baseline variation in these effects, although it has to be noted that these effects were tested under laboratory conditions only. Although greenhouse studies on the non-target effects of white cabbage cultivars were a good indicator for the effects that were observed in the field, field studies are always required in the final phase of the risk assessment procedure of transgenic plants to validate the results from the greenhouse. In the guidelines that we developed, we propose several novel aspects of testing for non-target effects of transgenic crops.

10.3 Future perspectives of genetic modification to protect crops

The use of transgenic crops in agriculture is expected to increase rapidly in the coming years (James 2010). It is predicted that not only new crop species will be transformed, but also that novel traits will be developed and that the use of stacked traits for resistance to multiple harmful organisms will increase (James 2010). Expected novel traits relate to resistance to biotic stresses (fungi, viruses and insects), resistance to abiotic stresses (drought, salt or cold), and the production of new compounds, such as medicines (Schuttelaar

& Partners et al. 2004; COGEM et al. 2010; James 2010). Especially the potential to develop transgenic crops that enhance biological control of pest herbivores by increasing the efficiency of natural enemies is exciting (**Chapter 2**; Degenhardt et al. 2003; Turlings and Ton 2006; Degenhardt et al. 2009). Studies using transgenic *A. thaliana* plants with a modified VOC emission show that the novel or increased emission of VOCs by genetic engineering can increase the attraction of predatory mites and parasitoid wasps (**Chapter 8**; Kappers et al. 2005; Beale et al. 2006; Schnee et al. 2006) and can repel herbivores (aphids and moths) (**Chapter 8**; Aharoni et al. 2003; Yang 2008). However, the results obtained with the model plant *A. thaliana* have to be validated with crop plants before transgenic plants with enhanced attraction of natural enemies can provide interesting new components for IPM. The studies by Degenhardt et al. (2003; 2009) on transgenic maize plants constitutively modified in their VOC emission are excellent examples of how this technique could be successfully applied in a crop plant. However, replacement of the constitutive promotor by an inducible promotor, for example one that is induced by herbivore feeding, will be essential for optimizing this transgenic technique (**Chapter 8**).

By incorporating transgenic crops in IPM, there are promising opportunities to avoid the potential ecological risks related to growing transgenic crops (**Chapter 2**). Although at present most studies report that the introduction of transgenic crops in agro-ecosystems has resulted in effects on non-target organisms that are within the range of effects observed for conventionally bred varieties, it remains of paramount importance that the effects of newly developed transgenic crops on non-target organisms are carefully assessed before a transgenic crop will be commercially cultivated.

10.4 Epilogue

Darwin wrote in his famous book *On the origin of species by means of natural selection* (1859): "*I am tempted to give one more instance showing how plants and animals, remote in the scale of nature, are bound together by a web of complex relations*". In the current thesis, several more examples of the complex interactions between plants and animals have been presented, not only for wild plants, but also for cultivated and genetically engineered plants. Plant resistance affected not only the performance, behaviour and abundance of herbivores, but also that of the natural enemies of these herbivores. A considerable intraspecific variation in the multitrophic effects of plant

resistance traits was observed, both among wild ecotypes and among cultivated varieties. The information on these multitrophic effects was used to explain the effects of transgenic plants modified in their direct and indirect resistance on the performance and behaviour of herbivores and carnivores. This thesis, combined with the other two theses within the ERGO-project (Houshyani 2012; Kabouw 2012), underlines the significance of using fundamental ecological knowledge in assessing the ecological effects of transgenic plants. It is expected that the information presented in this thesis will contribute to the development of an ecology-based protocol for assessing the effects of transgenic crops on non-target organisms. With the current fast technological developments in plant biotechnology, the urgency of additional ecological knowledge on future transgenic crops increases. We need to know more about the ecological effects of current and future transgenic crops on herbivores, carnivores, pollinators, decomposers and other organisms that are directly or indirectly associated with the transgenic crop. These ecological effects need to be studied not only on the short term, but especially on the long term to predict effects on the population dynamics of the organisms, the stability of the food web and consequentially the functioning of the entire ecosystem. In conclusion, in order to safely predict the effects of current and future transgenic crops, research on the complex ecological interactions between plants and aboveground and belowground biota, as well as on the mechanisms underlying these interactions, is indispensable.

10.5 Acknowledgements

I thank Joop van Loon, Marcel Dicke and Louise Vet for constructive comments on an earlier version of this chapter.

10

Agelopoulos NG, Dicke M and Posthumus MA (1995) Role of volatile infochemicals emitted by feces of larvae in host-searching behavior of parasitoid *Cotesia rubecula* (Hymenoptera: Braconidae): A behavioral and chemical study. *Journal of Chemical Ecology* 21: 1789-1811.

Agrawal AA, Janssen A, Bruin J, Posthumus MA and Sabelis MW (2002) An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. *Ecology Letters* 5: 377-385.

Agrawal AA and Kurashige NS (2003) A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *Journal of Chemical Ecology* 29: 1403-1415.

Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel WJ, Verstappen FWA, Verhoeven HA, Jongsma MA, Schwab W and Bouwmeester HJ (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell* 15: 2866-2884.

Aharoni A, Jongsma MA and Bouwmeester HJ (2005) Volatile science? Metabolic engineering of terpenoids in plants. *Trends in Plant Science* 10: 594-602.

Allison JD and Hare JD (2009) Learned and naive natural enemy responses and the interpretation of volatile organic compounds as cues or signals. *New Phytologist* 184: 768-782.

Almohamad R, Verheggen FJ and Haubruge E (2009) Searching and oviposition behavior of aphidophagous hoverflies (Diptera: Syrphidae): a review. *Biotechnologie Agronomie Societe et Environnement* 13: 467-481.

Anderson MJ and Legendre P (1999) An empirical comparison of permutation methods for tests of partial regression coefficients in a linear model. *Journal of Statistical Computation and Simulation* 62: 271-303.

Andow DA and Hilbeck A (2004) Science-based risk assessment for nontarget effects of transgenic crops. *Bioscience* 54: 637-649.

Andow DA and Zwahlen C (2006) Assessing environmental risks of transgenic plants. *Ecology Letters* 9: 196-214.

Andreasson E, Jorgensen LB, Hoglund AS, Rask L and Meijer J (2001) Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. *Plant Physiology* 127: 1750-1763.

Ankersmit GW, Dijkman H, Keuning NJ, Mertens H, Sins A and Tacoma HM (1986) *Episyrphus balteatus* as a predator of the aphid *Sitobion avenae* on winter wheat. *Entomologia Experimentalis et Applicata* 42: 271-277.

Aquilino KM, Cardinale BJ and Ives AR (2005) Reciprocal effects of host plant and natural enemy diversity on herbivore suppression: an empirical study of a model tritrophic system. *Oikos* 108: 275-282.

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

herbivore. *Chemoecology* 18: 65-71.

Aronson AI and Shai Y (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *Fems Microbiology Letters* 195: 1-8.

Bailey JK, Wooley SC, Lindroth RL and Whitham TG (2006) Importance of species interactions to community heritability: a genetic basis to trophic-level interactions. *Ecology Letters* 9: 78-85.

Baldwin IT, Halitschke R, Paschold A, von Dahl CC and Preston CA (2006) Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. *Science* 311: 812-815.

Bale JS, van Lenteren JC and Bigler F (2008) Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B-Biological Sciences* 363: 761-776.

Bangert RK, Allan GJ, Turek RJ, Wimp GM, Meneses N, Martinsen GD, Keim P and Whitham TG (2006) From genes to geography: a genetic similarity rule for arthropod community structure at multiple geographic scales. *Molecular Ecology* 15: 4215-4228.

Bardgett RD and Wardle DA (2010) *Aboveground-belowground linkages: biotic interactions, ecosystem processes, and global change*. Oxford University Press, Oxford, UK.

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T and Roberts J (2007) Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* 25: 1322-1326.

Beale MH, Birkett MA, Bruce TJA, Chamberlain K, Field LM, Huttly AK, Martin JL, Parker R, Phillips AL, Pickett JA, Prosser IM, Shewry PR, Smart LE, Wadhams LJ, Woodcock CM and Zhang YH (2006) Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. *Proceedings of the National Academy of Sciences of the United States of America* 103: 10509-10513.

Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabados L, Molthoff JW, Schipper B, Verbocht H, de Vos RCH, Morandini P, Aarts MGM and Bovy A (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. *Plos One* 3: e2068.

Benrey B, Callejas A, Rios L, Oyama K and Denno RF (1998) The effects of domestication of *Brassica* and *Phaseolus* on the interaction between phytophagous insects and parasitoids. *Biological Control* 11: 130-140.

Berenbaum MR and Zangerl AR (2008) Facing the future of plant-insect interaction research: Le Retour a la "Raison d'Etre". *Plant Physiology* 146: 804-811.

Bezemer TM, de Deyn GB, Bossinga TM, van Dam NM, Harvey JA and van der Putten WH (2005) Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology Letters* 8: 652-661.

Bezemer TM, Graca O, Rousseau P and van der Putten WH (2004) Above- and belowground trophic interactions on creeping thistle (*Cirsium arvense*) in high- and low-diversity plant communities: Potential for biotic resistance? *Plant Biology* 6: 231-238.

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

Bi RM, Jia HY, Feng DS and Wang HG (2006) Production and analysis of transgenic wheat (*Triticum aestivum* L.) with improved insect resistance by the introduction of cowpea trypsin inhibitor gene. *Euphytica* 151: 351-360.

Birch ANE, Griffiths BS, Caul S, Thompson J, Heckmann LH, Krogh PH and Cortet J (2007) The role of laboratory, glasshouse and field scale experiments in understanding the interactions between genetically modified crops and soil ecosystems: A review of the ECOGEN project. *Pedobiologia* 51: 251-260.

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

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

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

Bones A and Rossiter JT (2006) The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry* 67: 1053-1067.

Bouwmeester HJ (2006) Engineering the essence of plants. *Nature Biotechnology* 24: 1359-1361.

Bouwmeester HJ, Kappers IF, Verstappen FW, Aharoni A, Luckerhoff LLP, Lücker J, Jongsma MA and Dicke M (2003) Exploring multi-trophic plant-herbivore interactions for new crop protection methods. In *Proceedings of the International Congress Crop Science and Technology, Vol. 2, 10-12 November 2003, Glasgow, British Crop Protection Council, Alton, UK*, pp. 1123-1134.

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

Branquart E and Hemptinne JL (2000) Development of ovaries, allometry of reproductive traits and fecundity of *Episyrphus balteatus* (Diptera: Syrphidae). *European Journal of Entomology* 97: 165-170.

Bravo A and Soberon M (2008) How to cope with insect resistance to *Bt* toxins? *Trends in Biotechnology* 26: 573-579.

Bridges M, Jones AME, Bones AM, Hodgson C, Cole R, Bartlet E, Wallsgrove R, Karapapa VK, Watts N and Rossiter JT (2002) Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. *Proceedings of the Royal Society of London B Biological Sciences* 269: 187-191.

Broekgaarden C (2008) *An array of responses to insect feeding in Brassica.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Broekgaarden C, Poelman EH, Steenhuis G, Voorrips RE, Dicke M and Vosman B (2008) Responses of *Brassica oleracea* cultivars to infestation by the aphid *Brevicoryne brassicae*: an ecological and molecular approach. *Plant Cell and Environment* 31: 1592-1605.

Bruinsma M (2008) *Infochemical use in Brassica-insect interactions.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Bruinsma M and Dicke M (2008) Herbivore-induced indirect defense: from induction mechanisms to community ecology. In *Induced plant resistance to herbivory* (ed. by A Schaller), pp. 31-60. Springer, Dordrecht, The Netherlands.

Bruinsma M, Kowalchuk GA and van Veen JA (2003) Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils* 37: 329-337.

Buchner R (1987) Approach to determination of HPLC response factors for glucosinolates. In *Glucosinolates in rapeseeds* (ed. by JP Wathelet), pp. 50-58. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Bukovinszky T, Gols R, Posthumus MA, Vet LEM and van Lenteren JC (2005) Variation in plant volatiles and attraction of the parasitoid *Diadegma semiclausum* (Hellen). *Journal of Chemical Ecology* 31: 461-480.

Bukovinszky T, Poelman EH, Gols R, Prekatsakis G, Vet LEM, Harvey JA and Dicke M (2009) Consequences of constitutive and induced variation in plant nutritional quality for immune defence of a herbivore against parasitism. *Oecologia* 160: 299-308.

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

Burow M and Wittstock U (2009) Regulation and function of specifier proteins in plants. *Phytochemistry Reviews* 8: 87-99.

Carmona D, Lajeunesse MJ and Johnson MTJ (2011) Plant traits that predict resistance to herbivores. *Functional Ecology* 25: 358-367.

Carroll MJ, Schmelz EA, Meagher RL and Teal PEA (2006) Attraction of *Spodoptera frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *Journal of Chemical Ecology* 32: 1911-1924.

Carson R (1962) *Silent spring*. Fawcett Crest, New York, USA.

Carter N and Dixon AFG (1981) The 'natural enemy ravine' in cereal aphid population dynamics: a consequence of predator activity or aphid biology? *Journal of Animal*

Ecology 50: 605-611.

Caswell H and Hastings A (1980) Fecundity, developmental time, and population growth rate: an analytical solution. *Theoretical Population Biology* 17: 71-79.

Chadwick DJ and Marsh J (1993) *Crop protection and sustainable agriculture*. Wiley, Chichester, UK.

Chaplin-Kramer R, Kliebenstein DJ, Chiem A, Morrill E, Mills NJ and Kremen C (2011) Chemically mediated tritrophic interactions: opposing effects of glucosinolates on a specialist herbivore and its predators. *Journal of Applied Ecology* 48: 880-887.

Charleston DS and Dicke M (2008) *Designing experimental protocols to investigate the impact of GM crops on non-target arthropods.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Chen M, Zhao JZ, Shelton AM, Cao J and Earle ED (2008) Impact of single-gene and dual-gene *Bt* broccoli on the herbivore *Pieris rapae* (Lepidoptera: Pieridae) and its pupal endoparasitoid *Pteromalus puparum* (Hymenoptera: Pteromalidae). *Transgenic Research* 17: 545-555.

Chin HW and Lindsay RC (1993) Volatile sulfur compounds formed in disrupted tissue of different cabbage cultivars. *Journal of Food Science* 58: 835-839.

Clark BW, Phillips TA and Coats JR (2005) Environmental fate and effects of *Bacillus thuringiensis* (*Bt*) proteins from transgenic crops: a review. *Journal of Agricultural and Food Chemistry* 53: 4643-4653.

Clissold FJ, Sanson GD, Read J and Simpson SJ (2009) Gross vs. net income: How plant toughness affects performance of an insect herbivore. *Ecology* 90: 3393-3405.

Cloutier C, Boudreault S and Michaud D (2008) Impact of Colorado potato beetle-resistant potatoes on non-target arthropods: A meta-analysis of factors potentially involved in the failure of a *Bt* transgenic plant. *Cahiers Agricultures* 17: 388-394.

COGEM, CBD and Gezondheidsraad (2010) *Trendanalyse Biotechnologie 2009. Mondiaal momentum.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Cole RA (1997) The relative importance of glucosinolates and amino acids to the development of two aphid pests *Brevicoryne brassicae* and *Myzus persicae* on wild and cultivated brassica species. *Entomologia Experimentalis et Applicata* 85: 121-133.

Conner AJ, Glare TR and Nap JP (2003) The release of genetically modified crops into the environment - Part II. Overview of ecological risk assessment. *Plant Journal* 33: 19-46.

Cornelissen T and Stiling A (2006) Does low nutritional quality act as a plant defence? An experimental test of the slow-growth, high-mortality hypothesis. *Ecological Entomology* 31: 32-40.

Crutsinger GM, Collins MD, Fordyce JA, Gompert Z, Nice CC and Sanders NJ (2006) Plant genotypic diversity predicts community structure and governs an

ecosystem process. *Science* 313: 966-968.

Crutsinger GM, Reynolds WN, Classen AT and Sanders NJ (2008) Disparate effects of plant genotypic diversity on foliage and litter arthropod communities. *Oecologia* 158: 65-75.

Cunillera N, Arro M, Delourme D, Karst F, Boronat A and Ferrer A (1996) *Arabidopsis thaliana* contains two differentially expressed farnesyl-diphosphate synthase genes. *Journal of Biological Chemistry* 271: 7774-7780.

D'Alessandro M, Held M, Triponez Y and Turlings TCJ (2006) The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps. *Journal of Chemical Ecology* 32: 2733-2748.

Dabrowski ZT and Rodriguez JG (1971) Studies on resistance of strawberries to mites. 3. Preference and nonpreference responses of *Tetranychus urticae* and *T. turkestani* to essential oils of foliage. *Journal of Economic Entomology* 64: 387-391.

Darwin CR (1859) *On the origin of species by means of natural selection*. John Murray, London, UK.

Daun JK (2004) Quality of genetically modified (GM) and conventional varieties of canola (spring oilseed rape) grown in western Canada, 1996-2001. *Journal of Agricultural Science* 142: 273-280.

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

de Boer JG, Snoeren TAL and Dicke M (2005) Predatory mites learn to discriminate between plant volatiles induced by prey and nonprey herbivores. *Animal Behaviour* 69: 869-879.

de Maagd RA, Bosch D and Stiekema W (1999) *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends in Plant Science* 4: 9-13.

de Moel CP, Zwanepol S, Everaarts A, Alblas J and Hoek H (1996) *Teelt van sluitkool*. PAGV, Lelystad, The Netherlands.

de Moraes CM, Mescher MC and Tumlinson JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410: 577-580.

de Vos M and Jander G (2010) Volatile communication in plant-aphid interactions. *Current Opinion in Plant Biology* 13: 366-371.

de Vos M, Kim JH and Jander G (2007) Biochemistry and molecular biology of *Arabidopsis*-aphid interactions. *BioEssays* 29: 871-883.

Degenhardt J, Gershenzon J, Baldwin IT and Kessler A (2003) Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Current Opinion in Biotechnology* 14: 169-176.

Degenhardt J, Hiltpold I, Köllner TG, Frey M, Gierl A, Gershenzon J, Hibbard BE, Ellersieck MR and Turlings TCJ (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *Proceedings of the National Academy of Sciences of the United States of America* 106: 13213-13218.

Dent DR (1995) *Integrated pest management*. Chapman & Hall, London, UK.

Dicke M (1986) Volatile spider-mite pheromone and host-plant kairomone involved in spaced-out gregariousness in the spider mite *Tetranychus urticae*. *Physiological Entomology* 11: 251-262.

Dicke M (1999a) Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomologia Experimentalis et Applicata* 91: 131-142.

Dicke M (1999b) Direct and indirect effects of plants on performance of beneficial organisms. In *Handbook of Pest Management* (ed. by JR Ruberson), pp. 105-153. Marcel Dekker, New York, USA.

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

Dicke M and Hilker M (2003) Induced plant defences: from molecular biology to evolutionary ecology. *Basic and Applied Ecology* 4: 3-14.

Dicke M and Sabelis MW (1988) How plants obtain predatory mites as bodyguards. *Netherlands Journal of Zoology* 38: 148-165.

Dicke M, Sabelis MW, Takabayashi J, Bruin J and Posthumus MA (1990a) Plant strategies of manipulating predator-prey interactions through allelochemicals - Prospects for application in pest-control. *Journal of Chemical Ecology* 16: 3091-3118.

Dicke M, van Beek TA, Posthumus MA, Bendom N, van Bokhoven H and de Groot AE (1990b) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions - Involvement of host plant in its production. *Journal of Chemical Ecology* 16: 381-396.

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

Dicke M and van Poecke RMP (2002) Signalling in plant-insect interactions: signal transduction in direct and indirect plant defence. In *Plant signal transduction: Frontiers in Molecular Biology* (ed. by D Scheel and C Wasternack), pp. 289–316. Oxford University Press, Oxford, UK.

Dicke M, van Poecke RMP and de Boer JG (2003) Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic and Applied Ecology* 4: 27-42.

Dicke M and Vet LEM (1999) Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In *Herbivores: between plants and predators* (ed. by H Olff, VK Brown and RH Drent), pp. 483-520. Blackwell Science, Oxford, UK.

Doskotch RW, Cheng HY, Odell TM and Girard L (1980) Nerolidol: an antifeeding sesquiterpene alcohol for gypsy moth larvae from *Melaleuca leucadendron*. *Journal of Chemical Ecology* 6: 845-851.

Du YJ, Poppy GM, Powell W, Pickett JA, Wadhams LJ and Woodcock CM (1998) Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. *Journal of Chemical Ecology* 24: 1355-1368.

Duffey SS (1980) Sequestration of plant natural products by insects. *Annual Review of Entomology* 25: 447-477.

Dutton A, Romeis J and Bigler F (2003) Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: *Bt*-maize expressing Cry1Ab as a case study. *BioControl* 48: 611-636.

EFSA (2010) *Guidance on the environmental risk assessment of genetically modified plants.* European Food Safety Authority, Panel on Genetically Modified Organisms, Parma, Italy.

Eigenbrode SD (2004) The effects of plant epicuticular waxy blooms on attachment and effectiveness of predatory insects. *Arthropod Structure & Development* 33: 91-102.

Eisenhauer N, Horsch V, Moeser J and Scheu S (2010) Synergistic effects of microbial and animal decomposers on plant and herbivore performance. *Basic and Applied Ecology* 11: 23-34.

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

European Community (1990) Oilseeds-determination of glucosinolates High performance liquid chromatography. *Official Journal of the European Communities* L 170/28 Annex VIII: 03.07.27-34.

European Community (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of the European Communities* L 106/1.

Ferré J, van Rie J and MacIntosh SC (2008) Insecticidal genetically modified crops and Insect Resistance Management (IRM). In *Integration of insect-resistant genetically modified crops within IPM programs* (ed. by J Romeis, AM Shelton and GG Kennedy), pp. 41-86. Springer, Dordrecht, The Netherlands.

Fitt GP (2008) Have *Bt* crops led to changes in insecticide use patterns and impacted IPM? In *Integration of insect-resistant genetically modified crops within IPM programs* (ed. by J Romeis, AM Shelton and GG Kennedy), pp. 303-328. Springer, Dordrecht, The Netherlands.

Forkner RE and Hunter MD (2000) What goes up must come down? Nutrient addition and predation pressure on oak herbivores. *Ecology* 81: 1588-1600.

Francis F, Haubruge E, Hastir P and Gaspar C (2001a) Effect of aphid host plant on development and reproduction of the third trophic level, the predator *Adalia bipunctata* (Coleoptera: Coccinellidae). *Environmental Entomology* 30: 947-952.

Francis F, Lognay G, Wathelet JP and Haubruge E (2001b) Effects of

allelochemicals from first (Brassicaceae) and second (*Myzus persicae* and *Brevicoryne brassicae*) trophic levels on *Adalia bipunctata*. *Journal of Chemical Ecology* 27: 243-256.

Francis F, Lognay G, Wathelet JP and Haubruge E (2002) Characterisation of aphid myrosinase and degradation studies of glucosinolates. *Archives of insect biochemistry and physiology* 50: 173-182.

Gabrys B and Tjallingii WF (2002) The role of sinigrin in host plant recognition by aphids during initial plant penetration. *Entomologia Experimentalis et Applicata* 104: 89-93.

Gagic V, Tscharntke T, Dormann CF, Gruber B, Wilstermann A and Thies C (2011) Food web structure and biocontrol in a four-trophic level system across a landscape complexity gradient. *Proceedings of the Royal Society of London B Biological Sciences* 278: 2946-2953.

Gange AC and Brown VK (1989) Effects of root herbivory by an insect on a foliar-feeding species, mediated through changes in the host plant. *Oecologia* 81: 38-42.

Geervliet JBF (1997) *Infochemical use by insect parasitoids in a tritrophic context: comparison of a generalist and a specialist.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

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

Gigolashvili T, Yatusevich R, Berger B, Müller C and Flugge UI (2007) The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant Journal* 51: 247-261.

Godfray HCJ (1994) *Parasitoids: behavioral and evolutionary ecology*. Princeton University Press, Princeton, New Jersey, USA.

Gols R (2008) *Tritrophic interactions in wild and cultivated brassicaceous plant species.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Gols R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM and Harvey JA (2008a) Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. *Journal of Chemical Ecology* 34: 132-143.

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

Gols R, van Dam NM, Raaijmakers CE, Dicke M and Harvey JA (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? *Oikos* 118: 733-743.

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

Gouinguene S, Degen T and Turlings TCJ (2001) Variability in herbivore-induced

odour emissions among maize cultivars and their wild ancestors (teosinte). *Chemoecology* 11: 9-16.

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

Groot AT and Dicke M (2002) Insect-resistant transgenic plants in a multi-trophic context. *Plant Journal* 31: 387-406.

Groot MHM, van de Wiel CCM, van Tienderen PH and den Nijs HCM (2003) *Hybridization and introgression between crops and wild relatives. Current knowledge and research priorities in lieu of impending introductions of GM crops.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Haase J, Brandl R, Scheu S and Schadler M (2008) Above- and belowground interactions are mediated by nutrient availability. *Ecology* 89: 3072-3081.

Halaj J and Wise DH (2001) Terrestrial trophic cascades: How much do they trickle? *American Naturalist* 157: 262-281.

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

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

Hansen LC and Obrycki JJ (2000) Field deposition of *Bt* transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia* 125: 241-248.

Hare JD (1992) Effects of plant variation on herbivore-natural enemy interactions. In *Plant resistance to herbivores and pathogens: ecology, evolution, and genetics* (ed. by RS Fritz and EL Simms), pp. 278-198. The University of Chicago Press, Chicago, Illinois, USA.

Hare JD (2011) Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annual Review of Entomology* 56: 161-80.

Harvey JA (2005) Factors affecting the evolution of development strategies in parasitoid wasps: the importance of functional constraints and incorporating complexity. *Entomologia Experimentalis et Applicata* 117: 1-13.

Harvey JA, Kadash K and Strand MR (2000) Differences in larval feeding behavior correlate with altered developmental strategies in two parasitic wasps: implications for the size-fitness hypothesis. *Oikos* 88: 621-629.

Harvey JA, Poelman EH and Gols R (2010) Development and host utilization in *Hyposoter ebeninus* (Hymenoptera: Ichneumonidae), a solitary endoparasitoid of *Pieris rapae* and *P. brassicae* caterpillars (Lepidoptera: Pieridae). *Biological Control* 53: 312-318.

Harvey JA, van Dam NM and Gols R (2003) Interactions over four trophic levels: foodplant quality affects development of a hyperparasitoid as mediated through a

herbivore and its primary parasitoid. *Journal of Animal Ecology* 72: 520-531.

Hayton JC, Allen DG and Scarpello V (2004) Factor retention decisions in exploratory factor analysis: A tutorial on parallel analysis. *Organizational Research Methods* 7: 191-205.

Heil M (2004) Direct defense or ecological costs: Responses of herbivorous beetles to volatiles released by wild lima bean (*Phaseolus lunatus*). *Journal of Chemical Ecology* 30: 1289-1295.

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

Hellmich RL, Siegfried BD, Sears MK, Stanley-Horn DE, Daniels MJ, Mattila HR, Spencer T, Bidne KG and Lewis LC (2001) Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. *Proceedings of the National Academy of Sciences of the United States of America* 98: 11925-11930.

Henry CS, Brooks SJ, Duelli P and Johnson JB (2002) Discovering the true *Chrysoperla carnea* (Insecta: Neuroptera: Chrysopidae) using song analysis, morphology, and ecology. *Annals of the Entomological Society of America* 95: 172-191.

Hilker M and Meiners T (2011) Plants and insect eggs: How do they affect each other? *Phytochemistry* 72: 1612-1623.

Hoballah MEF, Tamo C and Turlings TCJ (2002) Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: Is quality or quantity important? *Journal of Chemical Ecology* 28: 951 -968.

Hoedjes KM, Kruidhof HM, Huigens ME, Dicke M, Vet LEM and Smid HM (2011) Natural variation in learning rate and memory dynamics in parasitoid wasps: opportunities for converging ecology and neuroscience. *Proceedings of the Royal Society of London B Biological Sciences* 278: 889-897.

Holmes MG and Keiller DR (2002) Effects of pubescence and waxes on the reflectance of leaves in the ultraviolet and photosynthetic wavebands: a comparison of a range of species. *Plant Cell and Environment* 25: 85-93.

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

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

Houshyani B (2012) *Application of omics technologies for environmental risk assessment of genetically modified plants, Arabidopsis and modified defence mechanisms as a model study.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Houshyani B, Kabouw P, Muth D, de Vos RCH, Bino RJ and Bouwmeester HJ (2012) Characterization of the natural variation in *Arabidopsis thaliana* metabolome by the analysis of metabolic distance. *Metabolomics* doi: 10.1007/s11306-011-0375-3.

Huang MS, Abel C, Sohrabi R, Petri J, Haupt I, Cosimano J, Gershenzon J and Tholl D (2010) Variation of herbivore-induced volatile terpenes among *Arabidopsis* ecotypes depends on allelic differences and subcellular targeting of two terpene synthases, TPS02 and TPS03. *Plant Physiology* 153: 1293-1310.

Hughes L and Bazzaz FA (2001) Effects of elevated CO2 on five plant-aphid interactions. *Entomologia Experimentalis et Applicata* 99: 87-96.

Hülskamp M and Schnittger A (1998) Spatial regulation of trichome formation in *Arabidopsis thaliana*. *Seminars in Cell & Developmental Biology* 9: 213-220.

Hunter MD and Price PW (1992) Playing chutes and ladders: heterogeneity and the relative roles of bottom-up and top-down forces in natural communities. *Ecology* 73: 724-732.

James C (2008) *Global status of commercialized biotech/GM crops: 2008.* International Service for the Acquisition of Agribiotech Applications (ISAAA), Ithaca, New York, USA.

James C (2010) *Global status of commercialized biotech/GM crops: 2010.* International Service for the Acquisition of Agribiotech Applications (ISAAA), Ithaca, New York, USA.

Johnson MTJ and Agrawal AA (2005) Plant genotype and environment interact to shape a diverse arthropod community on evening primrose (*Oenothera biennis*). *Ecology* 86: 874-885.

Johnson MTJ, Lajeunesse MJ and Agrawal AA (2006) Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. *Ecology Letters* 9: 24-34.

Jones AME, Bridges M, Bones AM, Cole R and Rossiter JT (2001) Purification and characterisation of a non-plant myrosinase from the cabbage aphid *Brevicoryne brassicae* (L.). *Insect Biochemistry and Molecular Biology* 31: 1-5.

Jones CG and Firn RD (1991) On the evolution of plant secondary chemical diversity. *Philosophical Transactions of the Royal Society B-Biological Sciences* 333: 273-280.

Jongsma MA, Gould F, Legros M, Yang LM, van Loon JJA and Dicke M (2010) Insect oviposition behavior affects the evolution of adaptation to *Bt* crops: consequences for refuge policies. *Evolutionary Ecology* 24: 1017–1030.

Jouanin L, Bonade-Bottino M, Girard C, Morrot G and Giband M (1998) Transgenic plants for insect resistance. *Plant Science* 131: 1-11.

Kabouw P (2012) *Consequences of intra-specific metabolic diversity in plants for soil organisms: A baseline approach for evaluating ecological effects of genetic modifications.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Kabouw P, Biere A, van der Putten WH and van Dam NM (2010a) Intra-specific differences in root and shoot glucosinolate profiles among white cabbage (*Brassica oleracea* var. *capitata*) cultivars. *Journal of Agricultural and Food Chemistry* 58: 411-417.

Kabouw P, Kos M, Kleine S, Vockenhuber EA, van Loon JJA, van der Putten WH, van Dam NM and Biere A (2011) Effects of soil organisms on aboveground multitrophic interactions are consistent between plant genotypes mediating the interaction. *Entomologia Experimentalis et Applicata* 139: 197-206 (**Chapter 5** in this thesis).

Kabouw P, van der Putten WH, van Dam NM and Biere A (2010b) Effects of intraspecific variation in white cabbage (*Brassica oleracea* var. capitata) on soil organisms. *Plant and Soil* 336: 509-518.

Kalaitzandonakes N (1999) A farm level perspective on agrobiotechnology: how much value and for whom? *AgBioForum* 2: 61-64.

Kalberer NM, Turlings TCJ and Rahier M (2001) Attraction of a leaf beetle (*Oreina cacaliae*) to damaged host plants. *Journal of Chemical Ecology* 27: 647-661.

Kalule T and Wright DJ (2004) The influence of cultivar and cultivar-aphid odours on the olfactory response of the parasitoid *Aphidius colemani*. *Journal of Applied Entomology* 128: 120-125.

Kappers IF, Aharoni A, van Herpen T, Luckerhoff LLP, Dicke M and Bouwmeester HJ (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. *Science* 309: 2070-2072.

Karban R (2011) The ecology and evolution of induced resistance against herbivores. *Functional Ecology* 25: 339-347.

Karban R and Baldwin IT (1997) *Induced Responses to Herbivory*. The University of Chicago Press, Chicago, Illinois, USA.

Karley AJ, Douglas AE and Parker WE (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology* 205: 3009-3018.

Kazana E, Pope TW, Tibbles L, Bridges M, Pickett JA, Bones AM, Powell G and Rossiter JT (2007) The cabbage aphid: a walking mustard oil bomb. *Proceedings of the Royal Society of London B Biological Sciences* 274: 2271-2277.

Kennedy GG (2008) Integration of insect-resistant genetically modified crops within IPM programs. In *Integration of insect-resistant genetically modified crops within IPM programs* (ed. by J Romeis, AM Shelton and KG G.), pp. 1-26. Springer, Dordrecht, The Netherlands.

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

Kessler A and Heil M (2011) The multiple faces of indirect defences and their agents of natural selection. *Functional Ecology* 25: 348-357.

Kim JH and Jander G (2007) *Myzus persicae* (green peach aphid) feeding on

Arabidopsis induces the formation of a deterrent indole glucosinolate. *Plant Journal* 49: 1008-1019.

Kim JH, Lee BW, Schroeder FC and Jander G (2008) Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *Plant Journal* 54: 1015-1026.

Kissen R, Pope TW, Grant M, Pickett JA, Rossiter JT and Powell G (2009) Modifying the alkylglucosinolate profile in *Arabidopsis thaliana* alters the tritrophic interaction with the herbivore *Brevicoryne brassicae* and parasitoid *Diaeretiella rapae*. *Journal of Chemical Ecology* 35: 958-969.

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J and Mitchell-Olds T (2001) Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiology* 126: 811-825.

Knols BGJ and Dicke M (2003) *Multitrofe interacties in genetisch gemodificeerde gewassen. Een enquête ter identificatie van belangrijke aandachtsvelden voor ecologisch onderzoek.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Koricheva J, Nykanen H and Gianoli E (2004) Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? *American Naturalist* 163: E64-E75.

Kortenhoff A (1993) Developments in the use of pesticides. In *Modern crop protection: developments and perspectives* (ed. by JC Zadoks), pp. 3-10. Wageningen Press, Wageningen, The Netherlands.

Kos M, Broekgaarden C, Kabouw P, Oude Lenferink K, Poelman EH, Vet LEM, Dicke M and van Loon JJA (2011a) Relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance on *Brassica oleracea*. *Functional Ecology* 25: 1113-1124 (**Chapter 4** in this thesis).

Kos M, Houshyani B, Achhami BB, Wietsma R, Gols R, Weldegergis BT, Kabouw P, Bouwmeester HJ, Vet LEM, Dicke M and van Loon JJA (2012) Herbivore-mediated effects of glucosinolates on different natural enemies of a specialist aphid. *Journal of Chemical Ecology* doi: 10.1007/s10886-012-0065-2 (**Chapter 6** in this thesis).

Kos M, Kabouw P, Noordam R, Hendriks K, Vet LEM, van Loon JJA and Dicke M (2011b) Prey-mediated effects of glucosinolates on aphid predators. *Ecological Entomology* 36: 377–388 (**Chapter 3** in this thesis).

Kos M, van Loon JJA, Dicke M and Vet LEM (2009) Transgenic plants as vital components of integrated pest management. *Trends in Biotechnology* 27: 621-627 (**Chapter 2** in this thesis).

Koschier EH, Sedy KA and Novak J (2002) Influence of plant volatiles on feeding damage caused by the onion thrips *Thrips tabaci*. *Crop Protection* 21: 419-425.

Koul O, Dhaliwal GS and W. CG (2004) *Integrated pest management: potential, constraints and challenges*. CABI, Wallingford, UK.

Kowalchuk GA, de Boer W and van Veen JA (2003) *Knowledge gaps with respect to the effects of genetically modified crops on the functioning of soil ecosystems.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA and Jeffery EH (1999) Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *Journal of Agricultural and Food Chemistry* 47: 1541-1548.

Kushad MM, Cloyd R and Babadoost MB (2004) Distribution of glucosinolates in ornamental cabbage and kale cultivars. *Scientia Horticulturae* 101: 215-221.

Lankau RA (2011) Intraspecific variation in allelochemistry determines an invasive species' impact on soil microbial communities. *Oecologia* 165: 453-463.

Lautenschlager GJ (1989) A comparison of alternatives to conducting Monte Carlo analyses for determining parallel analysis criteria. *Multivariate Behavioral Research* 24: 365-395.

Le Guigo P, Qu Y and Le Corff J (2011) Plant-mediated effects on a toxin-sequestering aphid and its endoparasitoid. *Basic and Applied Ecology* 12: 72-79.

Legendre P and Anderson MJ (1999) Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments (vol 69, pg 1, 1999). *Ecological Monographs* 69: 512-512.

Lewis WJ and Tumlinson JH (1988) Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331: 257-259.

Lewontin RC (1965) Selection for colonizing ability. In *The genetics of colonizing species* (ed. by HG Baker and GL Ledyard Stebbins), pp. 77-91. Academic Press, New York.

Li FF, Ye GY, Wu Q, Peng YF and Chen XX (2007) Arthropod abundance and diversity in *Bt* and non-*Bt* rice fields. *Environmental Entomology* 36: 646-654.

Losey JE, Rayor LS and Carter ME (1999) Transgenic pollen harms monarch larvae. *Nature* 399: 214-214.

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

Malone LA, Gatehouse AMR and Barratt BIP (2008) Beyond *Bt*: alternative strategies for insect-resistant genetically modified crops. In *Integration of insect-resistant genetically modified crops within IPM programs* (ed. by J Romeis, AM Shelton and KG G.), pp. 357-417. Springer, Dordrecht, The Netherlands.

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

Megias AG and Müller C (2010) Root herbivores and detritivores shape above-ground multitrophic assemblage through plant-mediated effects. *Journal of Animal Ecology* 79: 923-931.

Mewis I, Appel HM, Hom A, Raina R and Schultz JC (2005) Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiology* 138: 1149-1162.

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C and Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67: 2450-2462.

Miles CI, del Campo ML and Renwick JAA (2005) Behavioral and chemosensory responses to a host recognition cue by larvae of *Pieris rapae*. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* 191: 147-155.

Mithen R (2001) Glucosinolates - biochemistry, genetics and biological activity. *Plant Growth Regulation* 34: 91-103.

Müller C (2009) Interactions between glucosinolate- and myrosinase-containing plants and the sawfly *Athalia rosae*. *Phytochemistry Reviews* 8: 121-134.

Müller C, Agerbirk N, Olsen CE, Boeve JL, Schaffner U and Brakefield PM (2001) Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. *Journal of Chemical Ecology* 27: 2505-2516.

Müller C, Boeve JL and Brakefield P (2002) Host plant derived feeding deterrence towards ants in the turnip sawfly *Athalia rosae*. *Entomologia Experimentalis et Applicata* 104: 153-157.

Müller C and Brakefield PM (2003) Analysis of a chemical defense in sawfly larvae: Easy bleeding targets predatory wasps in late summer. *Journal of Chemical Ecology* 29: 2683-2694.

Müller R, de Vos M, Sun JY, Sonderby IE, Halkier BA, Wittstock U and Jander G (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *Journal of Chemical Ecology* 36: 905-913.

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

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

Nap JP, Metz PLJ, Escaler M and Conner AJ (2003) The release of genetically modified crops into the environment - Part I. Overview of current status and regulations. *Plant Journal* 33: 1-18.

Newton E, Bullock JM and Hodgson D (2009a) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. *Ecological Entomology* 34: 614-623.

Newton EL, Bullock JM and Hodgson DJ (2009b) Glucosinolate polymorphism in

wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. *Oecologia* 160: 63-76.

Ode PJ (2006) Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annual Review of Entomology* 51: 163-185.

Opitz SEW, Jensen SR and Müller C (2010) Sequestration of glucosinolates and iridoid glucosides in sawfly species of the genus *Athalia* and their role in defense against ants. *Journal of Chemical Ecology* 36: 148-157.

Orwin KH, Buckland SM, Johnson D, Turner BL, Smart S, Oakley S and Bardgett RD (2010) Linkages of plant traits to soil properties and the functioning of temperate grassland. *Journal of Ecology* 98: 1074-1083.

Papaj DR, Snellen H, Swaans K and Vet LEM (1994) Unrewarding experiences and their effect on foraging in the parasitic wasp *Leptopilina heterotoma* (Hymenoptera, Eucoilidae). *Journal of Insect Behavior* 7: 465-481.

Passardi F, Dobias J, Valerio L, Guimil S, Penel C and Dunand C (2007) Morphological and physiological traits of three major *Arabidopsis thaliana* accessions. *Journal of Plant Physiology* 164: 980-992.

Pike KS, Stary P, Miller T, Allison D, Graf G, Boydston L, Miller R and Gillespie R (1999) Host range and habitats of the aphid parasitoid *Diaeretiella rapae* (Hymenoptera : Aphidiidae) in Washington state. *Environmental Entomology* 28: 61-71.

Pimentel D (2002) *Encyclopedia of Pest Management*. Taylor & Francis Group, London, UK.

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

Poelman EH (2008) *Linking variation in plant defence to biodiversity at higher trophic levels: a multidisciplinary approach.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

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

Poelman EH, Galiart RJFH, Raaijmakers CE, van Loon JJA and van Dam NM (2008b) Performance of specialist and generalist herbivores feeding on cabbage cultivars is not explained by glucosinolate profiles. *Entomologia Experimentalis et Applicata* 127: 218-228.

Poelman EH, Gols R, Snoeren TAL, Muru D, Smid HM and Dicke M (2011) Indirect plant-mediated interactions among parasitoid larvae. *Ecology Letters* 14: 670-676.

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

Poelman EH, van Dam NM, van Loon JJA, Vet LEM and Dicke M (2009b) Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. *Ecology* 90: 1863-1877.

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

Poppy GM and Sutherland JP (2004) Can biological control benefit from genetically-modified crops? Tritrophic interactions on insect-resistant transgenic plants. *Physiological Entomology* 29: 257-268.

Poveda K, Steffan-Dewenter I, Scheu S and Tscharntke T (2003) Effects of below- and above-ground herbivores on plant growth, flower visitation and seed set. *Oecologia* 135: 601-605.

Prasifka JR, Hellmich RL, Dively GP, Higgins LS, Dixon PM and Duan JJ (2008) Selection of nontarget arthropod taxa for field research on transgenic insecticidal crops: Using empirical data and statistical power. *Environmental Entomology* 37: 1-10.

Pratt C (2008) Accumulation of glucosinolates by the cabbage aphid *Brevicoryne brassicae* as a defense against two coccinellid species. *Journal of Chemical Ecology* 34: 323-329.

Price DRG and Gatehouse JA (2008) RNAi-mediated crop protection against insects. *Trends in Biotechnology* 26: 393-400.

Price PW, Bouton CE, Gross P, McPheron BA, Thompson JN and Weis AE (1980) Interactions among 3 trophic levels - Influence of plants on interactions between insect herbivores and natural enemies. *Annual Review of Ecology and Systematics* 11: 41-65.

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

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

Read DP, Feeny PP and Root RB (1970) Habitat selection by the aphid parasite *Diaeretiella rapae* (Hymenoptera: Braconidae) and hyperparasite *Charips brassicae* (Hymenoptera: Cynipidae). *Canadian Entomologist* 102: 1567-1578.

Read J and Stokes A (2006) Plant biomechanics in an ecological context. *American Journal of Botany* 93: 1546-1565.

Reudler Talsma JH (2007) *Costs and benefits of iridoid glycosides in multitrophic systems.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Roitberg BD, Boivin G and Vet LEM (2001) Fitness, parasitoids, and biological control: an opinion. *Canadian Entomologist* 133: 429-438.

Romeis J, Meissle M and Bigler F (2006) Transgenic crops expressing *Bacillus*

thuringiensis toxins and biological control. *Nature Biotechnology* 24: 63-71.

Romeis J, Shelton AM and Kennedy GG (2008a) *Integration of insect-resistant genetically modified crops within IPM programs*. Springer, Dordrecht, The Netherlands.

Romeis J, van Driesche RG, Barratt BIP and Bigler F (2008b) Insect resistant transgenic crops and biological control. In *Integration of insect-resistant genetically modified crops within IPM programs* (ed. by J Romeis, AM Shelton and GG Kennedy), pp. 87-118. Springer, Dordrecht, The Netherlands.

Sadeghi A, Smagghe G, Broeders S, Hernalsteens JP, de Greve H, Peumans WJ and van Damme EJM (2008) Ectopically expressed leaf and bulb lectins from garlic (*Allium sativum* L.) protect transgenic tobacco plants against cotton leafworm (*Spodoptera littoralis*). *Transgenic Research* 17: 9-18.

Sanchez-Hernandez C, Lopez MG and Delano-Frier JP (2006) Reduced levels of volatile emissions in jasmonate-deficient *spr2* tomato mutants favour oviposition by insect herbivores. *Plant Cell and Environment* 29: 546-557.

Sarmah BK, Moore A, Tate W, Molvig L, Morton RL, Rees DP, Chiaiese P, Chrispeels MJ, Tabe LM and Higgins TJV (2004) Transgenic chickpea seeds expressing high levels of a bean alpha-amylase inhibitor. *Molecular Breeding* 14: 73 -82.

Saxena D and Stotzky G (2001) *Bt* corn has a higher lignin content than non-*Bt* corn. *American Journal of Botany* 88: 1704-1706.

Schmitz OJ, Hamback PA and Beckerman AP (2000) Trophic cascades in terrestrial systems: A review of the effects of carnivore removals on plants. *American Naturalist* 155: 141-153.

Schnee C, Kollner TG, Held M, Turlings TCJ, Gershenzon J and Degenhardt J (2006) The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proceedings of the National Academy of Sciences of the United States of America* 103: 1129-1134.

Scholte EJ and Dicke M (2005) *Effects of insect-resistant transgenic crops on non-target arthropods: first step in premarket risk assessment studies.* Commission on Genetic Modification, Bilthoven, The Netherlands.

Schoonhoven LM, van Loon JJA and Dicke M (2005) *Insect-plant Biology*. Oxford University Press, Oxford, UK.

Schuttelaar & Partners, Dicke M, van Tienderen PH and van Veen JA (2004) *Ecologisch onderzoek noodzakelijk voor de risicobeoordeling van toekomstige genetisch gemodificeerde planten.* Schuttelaar & Partners, Den Haag, The Netherlands.

Sell P and Kuosell HL (1990) Influence of infestation of oats by root-knot nematodes (*Meloidogyne* sp.) on the performance of the cereal aphid, *Metopolophium dirhodum* (Walk) (Hom Aphididae). *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* 109: 37-43.

Sequeira R and Mackauer M (1994) Variation in selected life-history parameters of the parasitoid wasp, *Aphidius ervi*: Influence of host developmental stage *Entomologia Experimentalis et Applicata* 71: 15-22.

Shelton AM and Sears MK (2001) The monarch butterfly controversy: scientific interpretations of a phenomenon. *Plant Journal* 27: 483-488.

Shelton AM, Zhao JZ and Roush RT (2002) Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. *Annual Review of Entomology* 47: 845-881.

Shiojiri K, Kishimoto K, Ozawa R, Kugimiya S, Urashimo S, Arimura G, Horiuchi J, Nishioka T, Matsui K and Takabayashi J (2006) Changing green leaf volatile biosynthesis in plants: An approach for improving plant resistance against both herbivores and pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 103: 16672-16676.

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

Sisterson MS, Biggs RW, Manhardt NM, Carriere Y, Dennehy TJ and Tabashnik BE (2007) Effects of transgenic *Bt* cotton on insecticide use and abundance of two generalist predators. *Entomologia Experimentalis et Applicata* 124: 305-311.

Smith CM (2005) *Plant resistance to arthropods: molecular and conventional approaches*. Springer, Dordrecht, The Netherlands.

Snoeren TAL (2009) *Herbivore-induced indirect defense of Arabidopsis.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Snoeren TAL, Kappers IF, Broekgaarden C, Mumm R, Dicke M and Bouwmeester HJ (2010) Natural variation in herbivore-induced volatiles in *Arabidopsis thaliana*. *Journal of Experimental Botany* 61: 3041-3056.

Snow AA, Andow DA, Gepts P, Hallerman EM, Power A, Tiedje JM and Wolfenbarger LL (2005) Genetically engineered organisms and the environment: Current status and recommendations. *Ecological Applications* 15: 377-404.

Soler R, Bezemer TM, van der Putten WH, Vet LEM and Harvey JA (2005) Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *Journal of Animal Ecology* 74: 1121-1130.

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

Soler R, Harvey JA, Rouchet R, Schaper SV and Bezemer TM (2010) Impacts of belowground herbivory on oviposition decisions in two congeneric butterfly species. *Entomologia Experimentalis et Applicata* 136: 191-198.

Stanley-Horn DE, Dively GP, Hellmich RL, Mattila HR, Sears MK, Rose R, Jesse LCH, Losey JE, Obrycki JJ and Lewis L (2001) Assessing the impact of Cry1Ab-

expressing corn pollen on monarch butterfly larvae in field studies. *Proceedings of the National Academy of Sciences of the United States of America* 98: 11931-11936.

Sznajder B and Harvey JA (2003) Second and third trophic level effects of differences in plant species reflect dietary specialisation of herbivores and their endoparasitoids. *Entomologia Experimentalis et Applicata* 109: 73-82.

Tabashnik BE, Gassmann AJ, Crowder DW and Carriere Y (2008) Insect resistance to *Bt* crops: evidence versus theory. *Nature Biotechnology* 26: 199-202.

Takabayashi J, Dicke M and Posthumus MA (1994) Volatile herbivore-induced terpenoids in plant mite interactions - variation caused by biotic and abiotic factors. *Journal of Chemical Ecology* 20: 1329-1354.

Takabayashi J, Sabelis MW, Janssen A, Shiojiri K and van Wijk M (2006) Can plants betray the presence of multiple herbivore species to predators and parasitoids? The role of learning in phytochemical information networks. *Ecological Research* 21: 3-8.

Tanaka S (2009) The impact of host aggressiveness on sex allocation by the gregarious parasitoid wasp *Cotesia glomerata* (L.). *Biology Letters* 5: 197-199.

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

Thies JE and Devare MH (2007) An ecological assessment of transgenic crops. *Journal of Development Studies* 43: 97-129.

Tjallingii WF and Hogen Esch TH (1993) Fine-structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiological Entomology* 18: 317-328.

Traw MB and Dawson TE (2002) Differential induction of trichomes by three herbivores of black mustard. *Oecologia* 131: 526-532.

Turlings TCJ and Ton J (2006) Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Current Opinion in Plant Biology* 9: 421-427.

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

Ulmer B, Gillott C and Erlandson M (2001) Feeding preferences, growth, and development of *Mamestra configurata* (Lepidoptera: Noctuidae) on Brassicaceae. *Canadian Entomologist* 133: 509-519.

van Bezooijen J (2006) *Methods and techniques for nematology. Course manual*. Wageningen University, Wageningen, The Netherlands.

van Dam NM and Oomen MWAT (2008) Root and shoot jasmonic acid applications differentially affect leaf chemistry and herbivore growth. *Plant Signaling & Behavior* 3: 91-98.

van Dam NM and Raaijmakers CE (2006) Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*.

Chemoecology 16: 17-24.

van Dam NM, Tytgat TOG and Kirkegaard JA (2009) Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews* 8: 171-186.

van Dam NM, Witjes L and Svatos A (2004) Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytologist* 161: 801-810.

van der Meijden E (1996) Plant defence, an evolutionary dilemma: Contrasting effects of (specialist and generalist) herbivores and natural enemies. *Entomologia Experimentalis et Applicata* 80: 307-310.

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

van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A and Stiekema WJ (1995) pBINPLUS: An improved plant transformation vector based on pBIN19. *Transgenic Research* 4: 288-290.

van Lenteren JC (1993) Integrated Pest Management: the inescapable trend. In *Modern crop protection: developments and perspectives* (ed. by JC Zadoks), pp. 217-225. Wageningen Press, Wageningen, The Netherlands.

van Lenteren JC (2008) *IOBC Internet book of biological control*. International Organization for Biological Control of Noxious Animals and Plants, Wageningen, The Netherlands.

van Lenteren JC (2012) The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. *BioControl* doi: 10.1007/ s10526-011-9395-1

van Leur H, Raaijmakers CE and van Dam NM (2008) Reciprocal interactions between the cabbage root fly (*Delia radicum*) and two glucosinolate phenotypes of *Barbarea vulgaris*. *Entomologia Experimentalis et Applicata* 128: 312-322.

van Loon JJA, Blaakmeer A, Griepink FC, van Beek TA, Schoonhoven LM and de Groot A (1992) Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3: 39 -44.

van Loon JJA, de Boer JG and Dicke M (2000) Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomologia Experimentalis et Applicata* 97: 219-227.

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

van Poecke RMP, Posthumus MA and Dicke M (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. *Journal of Chemical Ecology* 27: 1911-1928.

Vanhaelen N, Gaspar C and Francis F (2002) Influence of prey host plant on a generalist aphidophagous predator: *Episyrphus balteatus* (Diptera: Syrphidae). *European Journal of Entomology* 99: 561-564.

Verheggen FJ, Arnaud L, Bartram S, Gohy M and Haubruge E (2008) Aphid and plant volatiles induce oviposition in an aphidophagous hoverfly. *Journal of Chemical Ecology* 34: 301-307.

Verheggen FJ, Capella Q, Schwartzberg EG, Voigt D and Haubruge E (2009) Tomato-aphid-hoverfly: a tritrophic interaction incompatible for pest management. *Arthropod-Plant Interactions* 3: 141-149.

Vet LEM, de Jong AG, Franchi E and Papaj DR (1998) The effect of complete versus incomplete information on odour discrimination in a parasitic wasp. *Animal Behaviour* 55: 1271-1279.

Vet LEM and Dicke M (1992) Ecology of infochemical use by natural enemies in an tritrophic context. *Annual Review of Entomology* 37: 141-172.

Vet LEM, Lewis WJ and Cardé RT (1995) Parasitoid foraging and learning. In *Chemical ecology of insects* (ed. by W Bell and RT Cardé), pp. 65-101. Chapman and Hall, New York, USA.

von Burg S, van Veen FJF, Alvarez-Alfageme F and Romeis J (2011) Aphid-parasitoid community structure on genetically modified wheat. *Biology Letters* 7: 387 -391.

Vos M (2001) *Foraging under incomplete information: parasitoid behaviour and community dynamics.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Walker M, Hartley SE and Jones TH (2008) The relative importance of resources and natural enemies in determining herbivore abundance: thistles, tephritids and parasitoids. *Journal of Animal Ecology* 77: 1063-1071.

Wardle DA (2002) *Communities and ecosystems: linking the aboveground and belowground components*. Princeton University Press, Princeton, New Jersey, USA.

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH and Wall DH (2004a) Ecological linkages between aboveground and belowground biota. *Science* 304: 1629-1633.

Wardle DA, Yeates GW, Williamson WM, Bonner KI and Barker GM (2004b) Linking aboveground and belowground communities: the indirect influence of aphid species identity and diversity on a three trophic level soil food web. *Oikos* 107: 283-294.

Watkins MW (2000) *Monte Carlo PCA for Parallel Analysis*. State College, PA: Ed & Psych Associates.

Whitham TG, Young WP, Martinsen GD, Gehring CA, Schweitzer JA, Shuster SM, Wimp GM, Fischer DG, Bailey JK, Lindroth RL, Woolbright S and Kuske CR (2003) Community and ecosystem genetics: A consequence of the extended phenotype. *Ecology* 84: 559-573.

Williams IS (1999) Slow-growth, high-mortality - a general hypothesis, or is it? *Ecological Entomology* 24: 490-495.

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

Wurst S and Forstreuter M (2010) Colonization of *Tanacetum vulgare* by aphids is reduced by earthworms. *Entomologia Experimentalis et Applicata* 137: 86-92.

Wurst S, Langel R, Rodger S and Scheu S (2006) Effects of belowground biota on primary and secondary metabolites in *Brassica oleracea*. *Chemoecology* 16: 69-73.

Wurst S, van Dam NM, Monroy F, Biere A and van der Putten WH (2008) Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground herbivore damage. *Journal of Chemical Ecology* 34: 1360-1367.

Yang LM (2008) *Integration of host plant resistance and biological control: Using Arabidopsis-insect interactions as a model system.* PhD-thesis Wageningen University, Wageningen, The Netherlands.

Zhang XR, Henriques R, Lin SS, Niu QW and Chua NH (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* 1: 641-646.

Zwick WR and Velicer WF (1986) Comparison of 5 rules for determining the number of components to retain. *Psychological Bulletin* 99: 432-442.

Multitrophic effects of plant resistance traits

Plants play an essential role in mediating the interactions between a multitude of organisms. They can greatly influence the ecology and evolution of higher trophic levels, and they are important drivers of community structure and dynamics. Plants have evolved a wide array of direct and indirect plant resistance traits that prevent or reduce herbivory by insects, and there is considerable intraspecific variation in these traits within wild and cultivated plant species. Plants can affect the performance or behaviour of herbivores directly by chemical or physical means (direct resistance), or promote the effectiveness of natural enemies that feed on these herbivores (indirect resistance). Direct and indirect resistance strategies do not necessarily act independently, because secondary plant metabolites that confer direct resistance to herbivores can also influence the performance and behaviour of the natural enemies of these herbivores.

The aim of this thesis was to study the effects of direct and indirect plant resistance traits on the multitrophic interactions between plants, aboveground herbivores and their natural enemies. The following fundamental ecological research objectives were developed:

1) To study the effects of direct and indirect plant resistance traits on herbivores with different feeding strategies and/or levels of specialisation;
2) To study the effects of the same direct and indirect plant resistance traits on predators and parasitoids;

These objectives were addressed for plants in the family Brassicaceae, which contains many important crops and the model plant *Arabidopsis thaliana*. Brassicaceous plants produce several compounds that are involved in direct and indirect plant resistance, such as glucosinolates, their volatile breakdown products and other volatile compounds, and exhibit a large intraspecific variation in these traits. Insect species with different feeding strategies were selected for both the herbivores (phloem feeders and leaf chewers) and the natural enemies (parasitoids and predators), because these were expected to be differentially affected by (herbivore-mediated) plant resistance traits.

Four white cabbage (*Brassica oleracea*) cultivars that differ in direct and indirect resistance traits were used to address the objectives under greenhouse and field conditions. The specialist aphid *Brevicoryne brassicae* selectively sequestered glucosinolates from its host plant and used these for its own defence against predators (**Chapter 3**). The performance of two

predator species (the marmalade hoverfly, *Episyrphus balteatus* and the common green lacewing, *Chrysoperla carnea*) fed this aphid species was lower than when fed the non-sequestering generalist aphid *Myzus persicae*. When fed *B. brassicae* reared on different cultivars, predator performance seemed affected by specific glucosinolate profiles of the aphids.

In the field, the relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance was studied (**Chapter 4**). During two field seasons, the population dynamics of several herbivores (*Plutella xylostella* and *B. brassicae*) and carnivores (*E. balteatus*, *C. carnea*, *Diaeretiella rapae* and *Diadegma semiclausum*) were recorded. Intraspecific variation in plant chemistry and morphology consistently affected the abundance of insect herbivores and, consequently, their natural enemies. Differences in the fraction of herbivores parasitized and in predator oviposition on plants inoculated with a controlled number of herbivores could not explain the differences in abundance of the herbivores. It appeared that bottom-up forces (plant chemistry and morphology) were more important for herbivore abundance than plant-mediated top-down forces (attraction and arrestment of natural enemies).

It was examined whether the outcome of belowground–aboveground interactions can be affected by plant genotype (**Chapter 5**). Two white cabbage cultivars were selected, and the soil was inoculated with either nematodes or microorganisms and a sterilised soil acted as the control. The cultivar that sustained highest *B. brassicae* numbers also supported the best *D. rapae* performance. Microorganisms increased aphid population growth, but soil treatments did not affect parasitoid performance. Cultivars differed in several chemical and developmental traits, but showed similar responses of these traits to soil treatments. No interactions were found between cultivar and soil treatment for aphid population growth or parasitoid performance. Overall, the aboveground community was more affected by cultivar than by soil community.

Several wild-type and transgenic *A. thaliana* lines were used to address the objectives under laboratory conditions. In **Chapter 6 and 7** three *A. thaliana* ecotypes that differ in glucosinolate content and one transformed *A. thaliana* line with modified concentrations of aliphatic glucosinolates were used. The performance of *B. brassicae* was positively correlated with the concentrations of both aliphatic and indole glucosinolates in the phloem of the *A. thaliana* plants (**Chapter 6**). The aphid selectively sequestered

glucosinolates from the phloem, in agreement with the findings from **Chapter 3**. The glucosinolate concentration in *B. brassicae* was negatively correlated with the performance of *E. balteatus*, but positively with the performance of *D. rapae*, suggesting that the herbivore-mediated effects of glucosinolates differ between a predator and a parasitoid of this sequestering aphid. Both the predator and the parasitoid preferred the *A. thaliana* ecotype on which its offspring performed best, indicating a positive performance-preference correlation (**Chapter 6**).

For both the generalist leaf-chewer *Spodoptera exigua* and the specialist leaf-chewer *Pieris rapae* a negative correlation between performance and aliphatic glucosinolates concentrations was observed (**Chapter 7**), suggesting that even the specialist cannot completely inhibit the formation of toxic glucosinolate hydrolytic products. Surprisingly, the performance of a parasitoid of *P. rapae*, *Hyposoter ebeninus*, was positively correlated with higher concentrations of aliphatic glucosinolates in the plant, possibly caused by negative effects on host immune responses. This suggests that glucosinolates can not only confer resistance to herbivores directly, but also indirectly by increasing the performance of the parasitoids of these herbivores.

In **Chapter 8,** three *A. thaliana* ecotypes were transformed to emit one novel volatile compound and increased amounts of two endogenous compounds. *Brevicoryne brassicae* was repelled by the transgenic lines of two of the ecotypes, whereas its performance was not affected. *Diaeretiella rapae* preferred aphid-infested transgenic plants over aphid-infested wild-type plants for two of the ecotypes. In contrast, female *E. balteatus* predators did not discriminate between aphid-infested transgenic or wild-type plants. The results suggest that genetically engineering plants to modify their emission of volatiles holds considerable promise for improving control of herbivores, but the results have to be validated for a crop species before this approach can be applied in agriculture.

The results presented in **Chapters 3-8** indicate that there is a considerable intraspecific variation in the multitrophic effects of plant resistance traits both a) between cultivars of a crop species, and b) between plant ecotypes of a wild species. Plant resistance traits differentially affect the performance of phloem-feeding and leaf-chewing herbivores, and also differentially affect the performance and behaviour of natural enemies with different feeding strategies, *i.e.* predators and parasitoids. Direct and indirect

plant resistance traits were in conflict, but also worked in concert to enhance resistance to herbivores, depending on the biology of the herbivorous and carnivorous species involved.

From basic ecology to transgenic application

The knowledge that was gained during the fundamental ecological studies was applied to address the ecological effects of transgenic plants that are genetically engineered for an enhanced direct or indirect resistance against pest herbivores. Insect-resistant transgenic crops show clear benefits for agriculture and there are many new developments such as transgenic plants that enhance biological control. Transgenic crops may become vital components of Integrated Pest Management (**Chapter 2**). However, several ecological issues have to be considered before introducing transgenic crops into the environment, among which are the effects on beneficial non-target organisms, such as the natural enemies of pest herbivores. Although ecological knowledge was considered essential in the assessment of the environmental risks of transgenic crops, the acquisition of the data lagged behind the biotechnological developments. Therefore, in 2007, the Dutch government fostered a research programme aimed at strengthening the ecological risk analysis for transgenic plants: the ERGO (Ecology Regarding Genetically modified Organisms) programme. Its main objective was to study the ecology of transgenic crops to be able to develop ecology-based guidelines for assessing the effects of new transgenic crops. In accordance with the general aim of the ERGO-programme, the applied research objectives of this thesis were:

1) To assess baseline variation in effects of direct and indirect plant resistance traits on aboveground non-target organisms;
2) To compare the baseline variation with effects of transgenic plants altered in direct and indirect resistance traits on non-target organisms to assess whether transgenic effects exceed baseline variation;
3) To assess the validity of using greenhouse experiments to predict non-target effects in the field;
4) To develop guidelines for regulators to assess the effects of transgenic crops on non-target organisms in relation to baseline information, a combined effort with two other PhD-students.

The baseline variation refers to the variation in the effects on non-target organisms that already exists among conventional varieties of the crop

species, which is necessary to properly assess the ecological effects of a transgenic crop (**Chapter 9**). In this thesis, four white cabbage cultivars, as well as three *A. thaliana* ecotypes, were selected to represent the baseline variation. The selected cultivars and ecotypes differed considerably in plant resistance traits and consequently in effects on target and non-target organisms (**Chapters 3-8**), suggesting that these varieties and ecotypes represented a sufficiently broad range of non-target effects. The baseline variation in effects on target and non-target organisms was relatively consistent over different environments, soil types and time. The effects of transformed *A. thaliana* plants altered in direct and indirect resistance on non-target organisms were mostly not different from the baseline variation in these effects (**Chapter 10**). This indicates that the transformed plants were not disproportionally affecting aboveground non-target organisms compared to the wild ecotypes. Although greenhouse studies on the non-target effects of white cabbage cultivars provided good predictions of the effects that were observed in the field, it is suggested that field studies are always required in the final phase of the risk assessment procedure of transgenic plants to validate the results from the greenhouse (**Chapters 9 and 10**).

Finally, guidelines were developed that can be used to assess the effects of transgenic crops on non-target organisms (**Chapter 9)**. Several novel aspects of these guidelines relate to comparing non-target effects of transgenic crops with the baseline variation, representing this baseline variation correctly, and the value of using untargeted metabolomics and multivariate statistics.

This thesis underlines the significance of using fundamental ecological knowledge in assessing the ecological effects of transgenic plants. It is expected that the information presented in this thesis will contribute to the development of an ecology-based protocol for assessing the effects of transgenic crops on non-target organisms. With the current fast technological developments in plant biotechnology, the urgency of additional ecological knowledge on future transgenic crops increases. In order to safely predict the effects of current and future transgenic crops, research on the complex ecological interactions between plants and aboveground and belowground biota, as well as on the mechanisms underlying these interactions, is indispensable.

Multitrofe effecten van plantenverdediging

Planten spelen een essentiële rol in de interacties tussen een groot aantal organismen en kunnen de ecologie en de evolutie van organismen op hogere trofische niveaus, d.w.z. hoger in de voedselketen, sterk beïnvloeden. Ze spelen daarom een belangrijke rol in de structuur en de dynamiek van de gehele levensgemeenschap. Planten hebben een breed scala aan directe en indirecte verdedigingseigenschappen, die schade door planteneters voorkomen of verminderen. Er is een aanzienlijke variatie binnen één plantensoort in deze eigenschappen (ook wel intraspecifieke variatie genoemd), niet alleen binnen wilde plantensoorten maar ook binnen gecultiveerde soorten. Planten kunnen de overleving en groei en het gedrag van planteneters direct beïnvloeden door chemische of fysische eigenschappen (directe verdediging), of ze kunnen de effectiviteit van de natuurlijke vijanden van de planteneters verbeteren (indirecte verdediging). De directe en indirecte verdedigingseigenschappen van een plant werken niet per se onafhankelijk van elkaar. Plantenverdedigingsstoffen kunnen bijvoorbeeld ook de groei en het gedrag van de natuurlijke vijanden van deze planteneters beïnvloeden.

Het doel van de studie die gepresenteerd wordt in dit proefschrift was om de effecten van directe en indirecte verdedigingseigenschappen op de multitrofe interacties tussen planten, bovengrondse planteneters en hun natuurlijke vijanden te bestuderen. De volgende fundamentele ecologische onderzoeksdoelstellingen werden geformuleerd:

1) Het bestuderen van de effecten van directe en indirecte verdedigingseigenschappen op planteneters met verschillende voedingsstrategieën en/of specialisatieniveaus;
2) Het bestuderen van de effecten van dezelfde directe en indirecte verdedigings-eigenschappen op vijanden van planteneters, zoals roofinsecten en sluipwespen.

In deze studie werden planten in de familie Brassicaceae gebruikt, waartoe een aantal belangrijke gewassen zoals kool, spruitkool en mosterd en de modelplant *Arabidopsis thaliana* (de zandraket) behoren. Planten in deze familie produceren verschillende stoffen die betrokken zijn bij de directe en indirecte verdediging, zoals glucosinolaten, hun vluchtige afbraakproducten en andere vluchtige geurstoffen. Ze vertonen een grote intraspecifieke variatie in deze eigenschappen. In deze studie werden planteneters en natuurlijke vijanden met verschillende voedingsstrategieën geselecteerd, omdat werd

verwacht dat deze verschillend worden beïnvloed door de verdedigingseigenschappen. Binnen de planteneters werden floëemzuigers en bladvreters gekozen, en binnen de natuurlijke vijanden werden roofinsecten en sluipwespen geselecteerd. De larven van roofinsecten consumeren een groot aantal prooien tijdens hun ontwikkeling, terwijl de larven van sluipwespen zich binnen een enkele gastheer ontwikkelen en zich slechts met deze ene gastheer voeden.

Vier wittekoolcultivars (*Brassica oleracea*) die verschillen in directe en indirecte verdediging werden gebruikt in kas- en veldstudies. De gespecialiseerde bladluis *Brevicoryne brassicae* (melige koolluis) neemt de glucosinolaten uit de gastheerplant selectief op, en slaat deze op in zijn eigen lichaam. Dit opslaan van stoffen wordt 'sequestreren' genoemd. De bladluis gebruikt deze opgeslagen stoffen om zich te beschermen tegen zijn vijanden. De groei van twee roofinsecten, de zweefvlieg *Episyrphus balteatus* en de gaasvlieg *Chrysoperla carnea* die met deze bladluis werden gevoerd, was minder goed dan wanneer de roofinsecten werden gevoerd met de generalistische bladluis *Myzus persicae* die geen glucosinolaten sequestreert (**Hoofdstuk 3**). De groei van de roofinsecten leek ook te worden beïnvloed door de specifieke glucosinolaatprofielen in de bladluizen, welke afhankelijk bleken van de wittekoolcultivar waarop de bladluizen opgroeiden.

Het relatieve belang van de zogenaamde 'bottom-up' krachten (effecten van een plant op de planteneters) en 'top-down' krachten (effecten van een natuurlijke vijand op de planteneters) op de dichtheid van planteneters werd bestudeerd onder veldcondities (**Hoofdstuk 4**). De populatiedynamica van verschillende planteneters (de koolmot *Plutella xylostella* en de bladluis *B. brassicae*) en carnivoren (de roofinsecten *E. balteatus* en *C. carnea* en de sluipwespen *Diaeretiella rapae* en *Diadegma semiclausum*) werd gedurende twee veldseizoenen gevolgd. De intraspecifieke variatie in plantenchemie en -morfologie tussen de wittekoolcultivars beïnvloedde de dichtheid van plantenetende insecten, en als gevolg daarvan ook dat van hun natuurlijke vijanden. De effecten waren consistent over twee veldseizoenen. De verschillen in de dichtheid van de planteneters kon niet verklaard worden door de verschillen in aantrekking van sluipwespen of roofinsecten op planten waarop een vast aantal planteneters was gezet. Het bleek dat 'bottom-up' effecten, vooral van de chemie en morfologie van de plant, belangrijker waren voor het verklaren van de dichtheid van planteneters dan de 'top-down' effecten (de aantrekking van

natuurlijke vijanden).

Er werd onderzocht of de uitkomst van ondergrondse-bovengrondse interacties beïnvloed kan worden door het genotype van de plant (**Hoofdstuk 5**). Hiervoor werden twee wittekoolcultivars geselecteerd. De potgrond waarin de planten werden opgekweekt werd geïnoculeerd met nematoden (aaltjes) of micro-organismen (o.a. bacteriën), en steriele potgrond werd gebruikt als een controle-behandeling. De cultivar die de hoogste dichtheid aan *B. brassicae* bladluizen ondersteunde, ondersteunde ook de beste ontwikkeling van de sluipwesp van deze bladluis, *D. rapae*. Micro-organismen verhoogden de populatiegroei van de bladluizen, maar geen van de ondergrondse behandelingen beïnvloedde de ontwikkeling van de sluipwesp. De koolcultivars verschilden in meerdere chemische- en ontwikkelingseigenschappen, maar reageerden op een vergelijkbare manier op de bodembehandeling. Noch voor de populatiegroei van de bladluis, noch voor de ontwikkeling van de sluipwesp werden er interacties gevonden tussen cultivar en bodembehandeling. In het algemeen werd de bovengrondse insectengemeenschap meer beïnvloed door de cultivar dan door de bodembehandeling.

Verschillende *A. thaliana* lijnen werden gebruikt in laboratoriumexperimenten. In **Hoofdstuk 6 en 7** werden drie wilde accessies van *A. thaliana* die verschillen in glucosinolaatprofielen en een transgene lijn met een gemodificeerde concentratie aan alifatische glucosinolaten gebruikt. Glucosinolaten kunnen, op basis van het aminozuur waaruit ze zijn gemaakt en hun biologische effecten, worden verdeeld in alifatische en indoolglucosinolaten. De ontwikkeling van de bladluis *B. brassicae* was positief gecorreleerd met hogere concentraties van alifatische en indoolglucosinolaten in het floëem van de gastheerplant (**Hoofdstuk 6**). De bladluis sloeg selectief bepaalde glucosinolaten op in zijn lichaam, in overeenstemming met de resultaten uit **Hoofdstuk 3**. De concentratie van glucosinolaten in de bladluis was negatief gecorreleerd met de ontwikkeling van het roofinsect *E. balteatus*, maar positief met dat van de sluipwesp *D. rapae*. Dit suggereert dat de effecten van glucosinolaten, die via de bladluis werden doorgegeven aan de natuurlijke vijanden, verschillen tussen een roofinsect en een sluipwesp. In gedragstoetsen hadden het roofinsect en de sluipwesp allebei een voorkeur voor de *A. thaliana* accessie waarop hun nakomelingen het beste groeiden. Dit impliceert een positieve correlatie tussen groei en voorkeur voor beide insecten.

Voor zowel de generalistische mot *Spodoptera exigua* als de gespecialiseerde vlinder *Pieris rapae* werd een negatieve correlatie tussen de ontwikkelingsduur en de concentratie alifatische glucosinolaten gevonden (**Hoofdstuk 7**). Dit suggereert dat zelfs de specialist, die aangepast is aan het leven op planten die glucosinolaten bevatten, de vorming van toxische glucosinolaatafbraakproducten niet volledig kan voorkomen. Verrassend genoeg was de ontwikkeling van *Hyposoter ebeninus*, een sluipwesp van *P. rapae*, positief gecorreleerd met hogere concentraties aan glucosinolaten. Dit werd waarschijnlijk veroorzaakt door negatieve effecten van glucosinolaten op het immuunsysteem van *P. rapae*. Deze resultaten suggereren dat glucosinolaten niet alleen directe verdediging tegen planteneters kunnen veroorzaken, maar ook indirecte verdediging door de ontwikkeling en groei van sluipwespen te verbeteren.

In **Hoofdstuk 8** werden drie *A. thaliana* accessies bestudeerd die zodanig genetisch getransformeerd waren dat ze één nieuwe geurstof aanmaakten en een verhoogde productie lieten zien van twee bestaande geurstoffen. In gedragstoetsen werd de bladluis *B. brassicae* afgestoten door de transgene planten van twee van de accessies, terwijl zijn ontwikkelingsduur niet werd beïnvloed. De sluipwesp *D. rapae* had een voorkeur voor de transgene lijn, maar slechts voor twee van de drie accessies. In tegenstelling hiermee maakte de zweefvlieg *E. balteatus* geen keuze tussen transgene of wilde planten. Deze resultaten suggereren dat het genetisch transformeren van planten om hun productie van vluchtige stoffen te modificeren, een veelbelovende verdediging tegen plaaginsecten oplevert. Echter, de resultaten moeten eerst worden gevalideerd met een landbouwgewas voordat deze verdediging succesvol kan worden toegepast in de landbouw.

De resultaten in **Hoofdstuk 3-8** laten zien dat er een aanzienlijke intraspecifieke variatie is in effecten van de verdedigingseigenschappen van planten op planteneters en natuurlijke vijanden, a) tussen de cultivars van een landbouwgewas, en b) tussen de accessies van een wilde plant. De verdedigingseigenschappen beïnvloeden de ontwikkeling van floëemzuigende insecten anders dan die van bladvretende insecten. Bovendien beïnvloeden deze eigenschappen de ontwikkeling van roofinsecten en sluipwespen ook verschillend. De directe en indirecte verdedigingseigenschappen werkten in het ene geval elkaar tegen, maar werkten in het andere geval samen zodat de verdediging tegen planteneters verbeterde, afhankelijk van de biologie van de planteneter en de carnivoor.

Van ecologische basiskennis naar transgene toepassingen

De kennis die was gegenereerd tijdens de fundamentele ecologische studies werd toegepast om de ecologische effecten te onderzoeken van transgene planten die genetisch zijn gemodificeerd om een verhoogde directe of indirecte verdediging tegen plaaginsecten te verkrijgen. Transgene gewassen die resistent zijn tegen insecten hebben in de afgelopen jaren duidelijke voordelen voor de landbouw laten zien, en er zijn veel nieuwe ontwikkelingen zoals transgene planten die de biologische bestrijding van plaaginsecten bevorderen. Transgene planten zouden essentiële componenten van de geïntegreerde plaagbescherming kunnen worden (**Hoofdstuk 2**). Echter, verschillende ecologische aspecten moeten overwogen worden voordat deze transgene gewassen in landbouwsystemen kunnen worden geïntroduceerd. Een voorbeeld hiervan is het effect van deze gewassen op nuttige niet-doelorganismen (de organismen die niet het doel zijn van de plaagbestrijding), zoals de natuurlijke vijanden van plaaginsecten. Hoewel ecologische kennis van transgene gewassen als essentieel werd beschouwd om de risico's van transgene gewassen te kunnen beoordelen, liep de verwerving van deze kennis achter op de biotechnologische ontwikkelingen. In 2007 startte de Nederlandse overheid daarom een onderzoeksprogramma dat als doel had de ecologische risicoanalyse voor transgene gewassen te versterken: het ERGO-programma (ecologie omtrent genetisch gemodificeerde organismen). Het doel van dit programma was om de ecologie van transgene gewassen te bestuderen om te komen tot de ontwikkeling van richtlijnen gebaseerd op ecologische kennis om de effecten van nieuwe transgene gewassen te kunnen beoordelen. In overeenstemming met het doel van het ERGO-programma waren de toegepaste onderzoeksdoelstellingen van dit proefschrift als volgt:

1) Het inschatten van de 'baseline'-variatie in de effecten van directe en indirecte verdedigingseigenschappen van planten op bovengrondse niet -doelorganismen;
2) Het vergelijken van de 'baseline'-variatie met effecten van transgene gewassen op niet-doelorganismen om te beoordelen of de transgene effecten de 'baseline'-effecten overschrijden;
3) Het beoordelen van de geldigheid van het gebruik van kasexperimenten om de niet-doeleffecten in het veld te voorspellen;
4) Het ontwikkelen van richtlijnen om de effecten van transgene gewassen op niet-doelorganismen te beoordelen in relatie met de 'baseline'-

variatie, een gecombineerde inspanning met twee andere PhD-studenten.

De 'baseline'-variatie refereert naar de variatie in de effecten op niet-doelorganismen die reeds bestaat tussen conventionele cultivars van een gewas. Kennis van de 'baseline'-variatie is nodig om de ecologische effecten van transgene gewassen correct te kunnen inschatten (**Hoofdstuk 9**). In dit proefschrift werden vier wittekoolcultivars en drie *A. thaliana* accessies gebruikt om deze 'baseline'-variatie te representeren. De geselecteerde cultivars en accessies verschilden aanzienlijk in verdedigingseigenschappen en daardoor in de effecten op doel- (de plaaginsecten) en niet-doelorganismen (**Hoofdstukken 3-8**). Dit suggereert dat deze cultivars en accessies een voldoende brede variatie aan niet-doeleffecten representeerden. De 'baseline'-variatie in effecten op doel- en niet-doelorganismen was vrij consistent voor verschillende omgevingen, bodemtypes en de tijd. De effecten van de getransformeerde *A. thaliana* planten op niet-doelorganismen vielen grotendeels binnen de 'baseline'-variatie in deze effecten (**Hoofdstuk 10**). Dit laat zien dat de getransformeerde planten geen disproportioneel effect hadden op de bovengrondse niet-doelorganismen, vergeleken met de wilde accessies. Hoewel kasstudies een goede voorspellende waarde hadden voor de effecten die werden geobserveerd onder veldcondities, wordt aangeraden om veldstudies altijd uit te voeren in de laatste fasen van de risicoanalyse om de effecten gevonden onder kasomstandigheden te valideren (**Hoofdstukken 9 en 10**).

Tenslotte werden er richtlijnen ontwikkeld die gebruikt kunnen worden om de effecten van transgene gewassen op niet-doelorganismen te kunnen beoordelen (**Hoofdstuk 9**). Nieuwe aspecten in deze richtlijnen zijn: het vergelijken van de niet-doeleffecten van transgene gewassen met de 'baseline'-variatie, het correct representeren van deze 'baseline'-variatie, en het gebruik van 'metabolomics' (de studie van alle metabolieten in een organisme) en multivariate statistische methoden.

Dit proefschrift onderstreept het belang van fundamentele ecologische kennis in het beoordelen van de ecologische effecten van transgene planten. De verwachting is dat de informatie gepresenteerd in dit proefschrift zal bijdragen aan de ontwikkeling van een methode om inschattingen te maken van de ecologische effecten van transgene gewassen op niet-doelorganismen. Met de huidige snelle technologische ontwikkelingen neemt de noodzaak van kennis over de ecologie van toekomstige transgene gewassen toe. Onderzoek

naar de complexe ecologische interacties tussen planten en ondergrondse en bovengrondse organismen, evenals onderzoek naar de mechanismen die aan deze interacties ten grondslag liggen is daarvoor onmisbaar.

Aan het begin van je PhD-project denk je als kersverse PhD-student dat je alle tijd van de wereld hebt, maar de tijd vliegt, en ineens is het vier jaar later en is het zo ver: je proefschrift is klaar! Hoe snel het ook ging, dit proefschrift had niet tot stand kunnen komen zonder de steun van een groot aantal mensen. Allereerst wil ik de belangrijkste persoon in mijn leven bedanken: Arno. Je hebt me gemotiveerd, me op ideeën gebracht, en me door lastige perioden heen geholpen. Als ik 's avonds thuis kwam na een zware dag (of juist een hele inspirerende dag) had je altijd tijd voor het aanhoren van mijn frustraties of nieuwe ideeën, ook al kostte dat soms wel een uurtje. Je bent erg begripvol geweest als ik weer eens een weekend in de kas of het veld doorbracht. Vaak kwam je me zelfs helpen. Vele weekenden heb je met me doorgebracht met het tellen van minuscule bladluizen, duidelijk je favoriete werk.

Mijn begeleiders en (co-)promotors, Joop van Loon, Marcel Dicke en Louise Vet, wil ik bedanken voor hun inspanning in het tot stand komen van dit proefschrift. De kennis die jullie met me wilden delen was erg waardevol. Joop en Marcel, bedankt dat jullie deur altijd open stond. Of ik nou een vraag had, of op korte termijn jullie commentaar op een manuscript wilde ontvangen, jullie stonden voor me klaar. Joop, bedankt dat je me je kantoor niet uitgooide als ik voor de 5^{e} keer op een dag met een vraag binnenliep. Marcel, het verbaast me nog steeds dat je, als hoofd van een grote leerstoelgroep, meestal binnen één dag (zelfs op zaterdag) een manuscript van je waardevolle commentaar had voorzien. Louise, bedankt voor je inspiratie. Je bent mijn grote voorbeeld van een succesvolle vrouw in de wetenschap, en ik hoop dat we op het NIOO onze samenwerking kunnen voortzetten.

Benyamin Houshyani and Patrick Kabouw, thanks for the great collaboration during the last four years. You guys were vital for almost every part of this thesis, and we had many inspirational brainstorm sessions. Alle begeleiders binnen het ERGO project, Nicole van Dam, Arjen Biere, Wim van der Putten, en Harro Bouwmeester, bedankt voor de vruchtbare discussies die we hadden.

I wish to thank all the great colleagues that were at the lab of Ento during the last four years and that helped me a lot during my work, or to relax from work. Especially Marit, Tullu, Remco, Niels, Fedor, Janett, Maaike, Roland, Rieta, Patrick, Ana, Cindy, Dani, Colette, Erik, Tibor, Sander, Hans, Ties and Nina. You are the best colleagues someone could wish for. Nina, roomy, thanks for all the chats and support during our time in Radix, en Rieta, bedankt dat je altijd tijd voor me had, wanneer ik ook langskwam. A very

special thanks to Marit and Tullu, my old roomies and great friends, for making my entire PhD so memorable. I truly enjoyed the coffee breaks, parties, dinners, shopping sprees, weekend trips, sketch brainstorms, squash evenings and sarcasm matches. Niels & Anneke, Remco & Monique, Fedor & Ellen en Joke & Hans, bedankt dat jullie echte vrienden zijn geworden. Hoewel de meesten van jullie een andere baan hebben gevonden en Ento hebben verlaten, zien we elkaar nog vaak tijdens Vlaam-, squash- en spelletjes-avonden. Ik hoop dat nu ik bijna een dr. ben, dat jullie eindelijk kunnen ophouden me te plagen (ja, je weet tegen wie ik het heb!). Marit, Tullu and Remco, thanks for letting me practise for my own defence by supporting you on the stage as your paranymph. Marit en Anneke, bedankt dat jullie mijn eigen paranimfen zijn en me helpen de dag van mijn verdediging te overleven. Anneke en Niels, bedankt voor jullie hulp in het solliciteren voor mijn nieuwe baan op het NIOO en in het lay-outen van mijn thesis, maar bovenal bedankt voor jullie vriendschap (en niet te vergeten Annekes geweldige kookkunsten en het maken van zo'n enorm schattig o-larfje).

The six MSc-students whom I supervised, Koen Hendriks, Rozemarijn Noordam, Kirsten Oude Lenferink, Buddhi Achhami, Rafal Wietsma and Aart-Jan Overeem, thanks for your dedication. You have contributed to a large part of this thesis, and you taught me a lot.

All the members of the PhD council of the Graduate School Experimental Plant Sciences and Douwe Zuidema, Karin Horsman and Ingrid Vleghels, thanks for helping me develop myself into something more than 'just' a scientist. We had a lot of fun together. Unexpectedly, I truly enjoyed being the chairwoman of the council for a year, and we organised a whole lot of very interesting and fun gatherings for the PhD students of EPS.

De insectenkwekers, Léon Westerd, Frans van Aggelen, André Gidding en Leo Koopman, wil ik bedanken voor het mogelijk maken van mijn experimenten. Zonder de enorme aantallen insecten die jullie continue voor ons lab kweekten was dit proefschrift er niet geweest. Daarnaast wil ik de medewerkers van Unifarm, vooral André Maassen, Alex Super, Piet de Man, John van der Lippe en Rinie Verwoert, bedanken voor het opkweken van de duizenden planten en het onderhouden van mijn proefveld. Jullie hulp is onmisbaar geweest in mijn onderzoek.

Mijn lieve vriendinnen van de uni, Ilona, Renate, Marissa, Merel & Merel, en van thuis-thuis, Nienke, Vera, Marleen en Nicky, bedankt voor het geven van de hoognodige ontspanning gedurende de afgelopen vier jaar.

Mijn ouders en ideale schoonouders, bedankt voor het proberen te begrijpen wat ik bestudeerde, het enthousiasme als ik leuke resultaten had of een artikel had gepubliceerd, en het uitknippen van krantenartikelen die gerelateerd waren (of niet) aan mijn onderzoek. Mama, wat lief dat je vier jaar geleden al uit je hoofd hebt geleerd waar mijn onderzoek over ging, zodat je dat aan iedereen kon uitleggen. Joanne en Ted, Klaas en Anne en Joost en Carolien, bedankt voor de grapjes over mijn onderzoek zodat ik dat kon relativeren. Klaas en Anne, wat leuk dat jullie speciaal voor mij (toch??) terugkomen van jullie anderhalf jaar durende wereldreis. Jasper en Mathijs, jullie zijn de liefste neefjes die iemand zich kan wensen. Ik hoop dat papa en mama me vergeven dat ik jullie langzaam probeer om te vormen tot kleine bioloogjes (bij Jasper lijkt het zelfs te werken!).

Als één-na-laatste wil ik het 'Giant Cabbage' monument in Berastagi, Sumatra bedanken voor de inspiratie tijdens het schrijven van dit proefschrift. Wetende dat er serieus mensen zijn geweest die een monument hebben opgericht ter ere van een wittekoolplant heeft me elke dag weer lachend aan de slag doen gaan.

Als laatste wil ik Martijn Bezemer en Wim van der Putten van het NIOO bedanken voor hun vertrouwen in mij. De zekerheid van een interessante en uitdagende post-doc baan in het vooruitzicht heeft me de laatste maanden de benodigde rust gegeven om mijn proefschrift succesvol af te ronden. Ik kijk erg uit naar onze samenwerking de komende drie jaar.

Martine Kos was born on January 25th, 1984 in Oisterwijk, The Netherlands. After secondary school, she started her BSc study Biology at Wageningen University in 2002. During her MSc study Biology, specialisation Ecology, at the same university, she took part in research projects on the seasonal diet switch of impala and elephant (Kruger National Park, South Africa, 2006) and the usurpation of host behaviour by a parasitoid wasp (Wageningen University, 2007). Both MSc-theses led to a publication in a scientific journal. During her internship she studied the potential of the re-introduction of the red deer in The Netherlands (Stichting Brabants Landschap, 2007). Martine obtained her BSc and MSc degrees cum laude. She started her doctoral work on the multitrophic effects of plant resistance traits and the ecology of transgenic plants at the Laboratory of Entomology of the Wageningen University in 2007. Her research was part of the national research programme 'Ecology Regarding Genetically modified Organisms' funded by NWO. Martine published most of her thesis chapters in peer-reviewed scientific journals. She presented her thesis work at several national and international conferences, for instance in Sussex, England (British Ecological Society, 2010) and Perugia, Italy (International Organization of Biological Control, 2010). Furthermore, she was involved in the training and supervision of two BSc and six MSc students. Martine participated in the training programme of the national Graduate School Experimental Plant Sciences (EPS). She was a member of the EPS PhD Council and the EPS Educational Committee for over three years. From 2010-2011, she was the chairman of the EPS PhD Council. Martine organised several symposia and workshops, such as the 5th Plant-Insect Interactions workshop and the EPS ExPectationS Career day in 2010. From January 2012, Martine has continued her scientific career as a post-doc at the Netherlands Institute of Ecology (NIOO) in Wageningen, studying the belowground-aboveground interactions in ragwort (*Jacobaea vulgaris*) together with Martijn Bezemer.

Kos, M., Houshyani, B., Achhami, B.B., Wietsma, R., Gols, R., Weldegergis, BT., Kabouw, P., Bouwmeester, H.J., Vet, L.E.M., Dicke, M. and van Loon, J.J.A. **(2012)** Herbivore-mediated effects of glucosinolates on different natural enemies of a specialist aphid. *Journal of Chemical Ecology.* doi: 10.1007/s10886-012-0065-2

Kos, M., Houshyani, B., Wietsma, R., Kabouw, P., Vet, L.E.M., van Loon, J.J.A. and Dicke, M. **(2012)**. Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an associated parasitoid. *Phytochemistry.* doi: 10.1016/j.phytochem.2012.01.005

Kos M., Hoetmer A.J., Pretorius Y., de Boer W.F., de Knegt H., Grant C.C., Kohi E., Page B., Peel M., Slotow R., van der Waal C., van Wieren S.E., Prins H.H.T., van Langevelde F. **(2012)**. Seasonal diet changes in elephant and impala in mopane woodland. *European Journal of Wildlife Research.* doi: 10.1007/s10344-011-0575-1.

Broekgaarden, C., Riviere, P., Steenhuis, G., Cuenca, M., **Kos, M.** and Vosman, B. **(2012)**. Phloem-specific resistance in *Brassica oleracea* against the whitefly *Aleyrodes proletella*. *Entomologia Experimentalis et Applicata*. doi: 10.1111/j.1570-7458.2011.01210.x

Kos M., Broekgaarden C., Kabouw P., Oude Lenferink K., Poelman E.H., Vet L.E.M., Dicke M., van Loon J.J.A. **(2011)**. Relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance on *Brassica oleracea*. *Functional Ecology* 25: 1113-1124.

Kos M., Kabouw P., Noordam R., Hendriks K., Vet L.E.M., van Loon J.J.A., Dicke M. **(2011)**. Prey-mediated effects of glucosinolates on aphid predators. *Ecological Entomology* 36: 377-388.

Kabouw P.[*], **Kos M.[*],** Kleine S., Vockenhuber E.A., van Loon J.J.A., van der Putten W.H., van Dam N.M., Biere A. **(2011)**. Effects of soil organisms on aboveground multitrophic interactions are consistent between plant genotypes mediating the interaction. *Entomologia Experimentalis et Applicata* 139: 197-206. ***** *Both authors contributed equally*

Kos M., van Loon J.J.A., Dicke M., Vet L.E.M. **(2009)**. Transgenic plants as vital components of integrated pest management. *Trends in Biotechnology* 27: 621-627.

Harvey J.A., **Kos M.**, Nakamatsu Y., Tanaka T., Dicke M., Vet L.E.M., Brodeur J., Bezemer T.M. **(2008)**. Do parasitized caterpillars protect their parasitoids from hyperparasitoids? A test of the 'usurpation hypothesis'. *Animal Behaviour* 76: 701-708.

Submitted

Kos, M., Houshyani, B., Overeem, A.J., Bouwmeester, H.J., Weldegergis, BT., van Loon, J.J.A., Dicke, M. and Vet, L.E.M. Genetic engineering of plant volatile terpenoids: effects on a herbivore, a predator and a parasitoid.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Martine Kos**
Date: **16 March, 2012**
Group: **Laboratory of Entomology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Ecological effects of plant resistance traits on above-ground non-target organisms in different genotypes of *Brassica* and *Arabidopsis*	Jan 29, 2008
► **Writing or rewriting a project proposal**	
Ecological effects of plant resistance traits on above-ground non-target organisms in different genotypes of *Brassica* and *Arabidopsis*	Nov 2007
► **Writing a review or book chapter**	
Transgenic plants as vital components of integrated pest management, *Trends in Biotechnology (2009) 27, 621-627*	May 2009
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
First Joint Retreat of PhD Students in Plant Sciences, Wageningen	Oct 02-03, 2008
EPS PhD Students day, Leiden	Feb 26, 2009
EPS PhD Students day, Utrecht	Jun 01, 2010
EPS PhD Students day, Wageningen	May 20, 2011
► **EPS theme symposia**	
EPS theme 2, Utrecht	Jan 22, 2009
EPS theme 2, Utrecht	Jan 15, 2010
► **NWO Lunteren days and other National Platforms**	
Netherlands Entomological Society Annual Meeting, Ede	Dec 14, 2007
NERN Annual Meeting, Lunteren	Feb 12-13, 2008
National Ecogenomics day, Ede	Feb 29, 2008
NERN Annual Meeting, Lunteren	Feb 10-11, 2009
NERN Annual Meeting, Lunteren	Feb 09-10, 2010
Netherlands Entomological Society Annual Meeting, Ede	Dec 17, 2010
NERN Annual Meeting, Lunteren	Feb 08-09, 2011
► **Seminars (series), workshops and symposia**	
KNPV-najaarsvergadering, Wageningen	Dec 13, 2007
Local monthly seminars Entomology, Wageningen	2007-2011
Symposium: Plant interactions with aphids, Wageningen	Aug 19-20 2008
3rd workshop Plant-Insect Interactions, Leiden	Oct 29, 2008
Symposium: Current Themes in Ecology: 'Plants - insects - microbes', Wageningen	Nov 07, 2008
Symposium 'Ecology Regarding Genetically Modified Organisms' part I, Utrecht	Jun 30, 2009
EPS symposium Ecology and Experimental Plant Sciences 2, Wageningen	Sep 22, 2009
4rd workshop Plant-Insect Interactions, Utrecht	Nov 11, 2009
Symposium 'Ecology Regarding Genetically Modified Organisms' part II, Utrecht	Feb 25-26, 2010
EPS Lunteren days, Lunteren	Apr 19-20, 2010
Yearly Entomology Research Exchange Meeting, Renkum	Jun 15, 2010
5th workshop Plant-Insect Interactions, Wageningen	Nov 11, 2010
EPS ExPectationS Career day, Wageningen	Nov 19, 2010
Yearly Entomology Research Exchange Meeting, Renkum	Jun 07, 2011
► **Seminar plus**	

►	**International symposia and congresses**	
	British Ecological Society annual symposium: 'Secondary metabolites', England	Apr 12-14, 2010
	Symposium IOBC 'Ecology of Aphidophaga', Italy	Sep 20-25, 2010
	Contribution from scientific research to the risk assessment of GMOs', Belgium	Oct 21-22, 2010
	International Symposium on Insect-Plant Interactions, Netherlands	Aug 13-18, 2011
►	**Presentations**	
	Poster and Oral presentation, First Joint Retreat of PhD students in Plant Sciences, Wageningen	Oct 02-03, 2008
	Poster NERN, Lunteren / 4rd workshop Plant-Insect Interactions, Utrecht	Feb 10-11, 2009/Nov 11, 2009
	Oral presentation EPS theme 2 day, Utrecht	Jan 15, 2010
	Poster, British Ecological Society annual symposium: 'Secondary metabolites', England	Apr 12-14, 2010
	Oral presentation EPS Lunteren days, Lunteren	Apr 19-20, 2010
	Oral presentation IOBC Symposium 'Ecology of Aphidophaga', Italy	Sept 20-24, 2010
	Oral presentation Netherlands Entomological Society Annual Meeting, Ede	Dec 17, 2010
	Poster, International Symposium on Insect-Plant Interactions, Wageningen	Aug 13-18, 2011
►	**IAB interview**	Feb 17, 2011
►	**Excursions**	
	Subtotal Scientific Exposure	*25.5 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Winter School: 'Ecology of Plant Volatile Organic Compounds', PE&RC, Wageningen	Nov 12-14, 2008
	Basic statistics, PE&RC, Wageningen	Dec 15-19, 2008
	Linear Mixed Models, PE&RC, Wageningen	May 25-26, 2009
	Multivariate Statistics, Umetrics, Wageningen	Oct 26-28, 2009
	Generalized Linear Models, PE&RC, Wageningen	Jun 28-29, 2010
	Parasitoid Biology Course, NIOO, Wageningen	Dec 14-15, 2010
►	**Journal club**	
	PHD journal club Entomology	2007-2011
►	**Individual research training**	
	Subtotal In-Depth Studies	*8.1 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD Competence Assessment, EPS, Wageningen	Feb 19, 2008
	Techniques for Writing and Presenting a Scientific Paper, SENSE, Wageningen	Aug 31-Sep 03, 2010
	Minisymposium How to write a world class paper, WUR Library, Wageningen	Oct 26, 2010
►	**Organisation of PhD students day, course or conference**	
	Organisation of 5th workshop Plant-Insect Interactions, Wageningen	Nov 11, 2010
	Organisation of EPS ExPectationS Career day, Wageningen	Nov 19, 2010
►	**Membership of Board, Committee or PhD council**	
	EPS PhD-council and Educational Committee	2008-2011
	Chairman EPS PhD-council	2010-2011
	Subtotal Personal Development	*7.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**54,7**

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

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

Table S4.1. Description of the binomially distributed dependent variable ('variable') and fixed binomial totals ('totals) used in the Generalized Linear Mixed Model (GLMM) or Generalized Linear Model (GLM) analyses performed in this study.

Experiment	Analysis of	Variable	Totals
Insect abundance (GLMM)	Fraction of naturally occurring *Plutella xylostella* caterpillars that were parasitized	Number of *D. semiclausum* pupae	Summed numbers of *P. xylostella* caterpillars, pupae and *D. semiclausum* pupae
	Fraction of naturally occurring *Brevicoryne brassicae* aphids that were parasitized	Number of *D. rapae* mummies with *B. brassicae* as host	Summed numbers of *B. brassicae* aphids and *D. rapae* mummies with *B. brassicae* as host
	Fraction of naturally occurring other aphids that were parasitized	Number of *D. rapae* mummies with other aphids as host	Summed numbers of other aphids and *D. rapae* mummies with other aphids as host
Parasitisation and oviposition (GLM)	Fraction of experimentally released *P. xylostella* caterpillars that were recovered from the field	Number of recovered caterpillars	Number of released caterpillars
	Fraction of experimentally released *B. brassicae* aphids that were recovered from the field	Number of recovered aphids	Number of released aphids
	Fraction of *P. xylostella* caterpillars that were parasitized	Number of *D. semiclausum* pupae	Summed numbers of parasitized and unparasitized caterpillars
	Fraction of *B. brassicae* aphids that were parasitized	Number of *D. rapae* mummies	Summed numbers of parasitized and unparasitized aphids
	Fraction of *D. semiclausum* adults that were female	Number of *D. semiclausum* females	Number of adult parasitoids

Table S4.2. Effects of plant cultivar, time and their interaction on the abundance of several insect species on *Brassica oleracea* plants in the field, as analysed by repeated measures mixed models.

		Cultivar (*d.f.* = 3)		Time (*d.f.* = 13)		Cultivar x Time (*d.f.* = 39)	
Species and dev. stage	**Year**	**F**[a]	***P***	**F**	***P***	**F**	***P***
Plutella xylostella caterpillars	2008	6.92	**< 0.001**[b]	98.78	**< 0.001**	1.35	0.095
	2009	51.69	**< 0.001**	229.53	**< 0.001**	3.62	**< 0.001**
Plutella xylostella pupae	2008	1.47	0.227	46.10	**< 0.001**	1.22	0.190
	2009	9.51	**< 0.001**	23.44	**< 0.001**	1.01	0.461
Diadegma semiclausum pupae	2008	3.69	**0.015**	65.39	**< 0.001**	1.93	**0.001**
	2009	60.84	**< 0.001**	198.04	**< 0.001**	3.95	**< 0.001**
Brevicoryne brassicae aphids	2008	43.82	**< 0.001**	32.88	**< 0.001**	2.59	**< 0.001**
	2009	93.33	**< 0.001**	14.69	**< 0.001**	4.51	**< 0.001**
Other aphids	2008	17.01	**< 0.001**	81.58	**< 0.001**	0.85	0.719
	2009	61.20	**< 0.001**	88.01	**< 0.001**	5.55	**< 0.001**
Diaeretiella rapae (*B. brassicae* mummies)	2008	24.14	**< 0.001**	34.45	**< 0.001**	2.52	**< 0.001**
	2009	30.44	**< 0.001**	11.88	**< 0.001**	2.88	**< 0.001**
Diaeretiella rapae (other aphid mummies)[c]	2009	14.78	**< 0.001**	18.030	**< 0.001**	3.28	**< 0.001**
Episyrphus balteatus larvae	2008	15.28	**< 0.001**	24.07	**< 0.001**	2.43	**< 0.001**
	2009	21.89	**< 0.001**	3.15	**< 0.001**	1.59	**0.020**

Episyrphus balteatus pupae[a]	2008	34.53	**< 0.001**	8.22	**< 0.001**	4.52	**< 0.001**
Other predatory hoverfly larvae[a]	2008	8.92	**< 0.001**	8.95	**< 0.001**	1.50	**0.037**
Other predatory hoverflies pupae[a]	2008	7.10	**< 0.001**	3.47	**< 0.001**	1.42	0.061
Chrysoperla carnea eggs	2008	1.25	0.293	25.40	**< 0.001**	1.14	0.280
	2009	7.65	**< 0.001**	10.61	**< 0.001**	1.82	**0.004**

[a] F-values were rounded off to two decimals. [b] Boldface values represent significant effects at P < 0.05. [c] Analysis of D. rapae that parasitized other aphids, E. balteatus pupae and other predatory hoverfly larvae and pupae were not performed for the data obtained in 2009 because numbers were too low for meaningful statistical analyses.

Table S4.3. Effects of plant cultivar, time and their interaction on the fraction of parasitized herbivores naturally occurring on *Brassica oleracea* plants in field plots, as analysed by Generalized Linear Mixed Models.

		Cultivar		Time[a]		Cultivar x Time	
Fraction parasitism	**Year**	**F** [b]	***P*** [c]	**F**	***P***	**F**	***P***
Fraction of *Plutella xylostella* caterpillars	2008	0.59 (3; 25.5)	0.630	56.82 (3; 84.2)	**< 0.001**	1.51 (9; 84.1)	0.156
parasitized	2009	3.66 (3; 26.1)	**0.025**	107.93 (6; 167.8)	**< 0.001**	1.50 (18; 167.8)	0.096
Fraction of *Brevicoryne brassicae* aphids	2008	1.34 (3; 29.4)	0.280	17.11 (8; 198.0)	**< 0.001**	1.44 (24; 198.4)	0.094
parasitized	2009	3.31 (3; 24.0)	**0.037**	9.68 (3; 73.9)	**< 0.001**	1.37 (9; 75.6)	0.215
Fraction of other aphids parasitized[d]	2009	2.56 (3; 36.5)	0.070	12.61 (5; 141.0)	**< 0.001**	1.03 (15; 141.0)	0.425

[a] The number of weeks included in the analysis was determined separately for every analysis, based on the number of herbivores present (as described in the Methods). [b] F-values were rounded off to two decimals. Between brackets the degrees of freedom (numerator d.f.; denominator d.f.). [c] Significance was evaluated by means of Wald F-test. Boldface values represent significant effects at P < 0.05. [d] Analysis of the fraction of other aphids parasitized was not performed in 2008 as numbers were too low for meaningful statistical analysis

Table S4.4. Average fraction (mean ± SE) of naturally occurring herbivores that were parasitized on plants of four *Brassica oleracea* cultivars in field plots

		***Brassica oleracea* cultivar**			
		Rivera	**Lennox**	**Christmas Drumhead**	**Badger Shipper**
Fraction parasitism	**Year**				
Fraction of *Plutella xylostella* caterpillars parasitized	2008 (4)[a]	0.32 ± 0.04	0.36 ± 0.05	0.32 ± 0.05	0.32 ± 0.04
	2009 (7)	0.28 ± 0.03	0.26 ± 0.02	0.27 ± 0.02	0.23 ± 0.02
Fraction of *Brevicoryne brassicae* aphids parasitized	2008 (9)	0.15 ± 0.02	0.12 ± 0.02	0.11 ± 0.01	0.11 ± 0.02
	2009 (4)	0.32 ± 0.11	0.29 ± 0.07	0.10 ± 0.03	0.12 ± 0.04
Fraction of other aphids parasitized[b]	2009 (6)	0.19 ± 0.04	0.08 ± 0.02	0.08 ± 0.02	0.09 ± 0.03

[a] Between brackets the number of weeks for which the average was calculated, which was determined based on the number of herbivores present (as described in the Methods). [b] Calculation of the average fraction of other aphids parasitized in 2008 was not performed, as numbers of other aphids were too low for meaningful calculations.

Table S4.5. Effects of plant cultivar, leaf age and their interaction on chemical and morphological traits of plants of four *Brassica oleracea* cultivars grown under field conditions

	Cultivar (*d.f.* = 3)		Leaf age (*d.f.* = 2)		Cultivar x leaf age (*d.f.* = 6)	
2008[a]	F	*P*	F	*P*	F	*P*
Indole GLS[b]	4.984	**0.007**[c]	n.a.[d]	n.a.	n.a.	n.a.
Aliphatic GLS[b]	1.388	0.268	n.a.	n.a.	n.a.	n.a.
Total GLS	0.941	0.435	n.a.	n.a.	n.a.	n.a.
Total amino acids	0.759	0.527	n.a.	n.a.	n.a.	n.a.
Total sugars	1.060	0.382	n.a.	n.a.	n.a.	n.a.
2009						
Indole GLS[b]	5.09	**0.003**	49.53	**< 0.001**	3.04	**0.010**
Aliphatic GLS[b]	7.60	**< 0.001**	107.87	**< 0.001**	3.11	**0.008**
Total GLS	8.48	**< 0.001**	104.23	**< 0.001**	3.30	**0.006**
Fresh weight shoot	3.77	**0.022**	n.a.	n.a.	n.a.	n.a.
Epicuticular wax[e]	9.00	**0.029**	n.a.	n.a.	n.a.	n.a.
Leaf toughness	141.00	**< 0.001**	31.16	**< 0.001**	7.36	**< 0.001**
Reflectance (%) at 330 nm	2.57	0.060	28.61	**< 0.001**	3.09	**0.009**
Reflectance (%) at 550 nm	57.95	**< 0.001**	35.64	**< 0.001**	1.21	0.301
Reflectance (%) at 680 nm	77.60	**< 0.001**	3.23	**0.044**	3.50	**0.004**

[a] In 2008 only intermediate-aged leaves were sampled. [b] Glucosinolates (GLS) were grouped according to their biosynthetic origin. [c] Boldface values represent significant effects at P < 0.05. [d] n.a. = not assessed (trait not quantified for different leaf ages). [e] Epicuticular wax was collected only from intermediate-aged leaves, and analysed by Kruskal-Wallis H-tests as assumptions of normality were violated.

Table S4.6. Effects of plant cultivar, time and their interaction on the fraction of experimentally inoculated herbivores that were recovered from plants of four *Brassica oleracea* cultivars, as analysed by Generalized Linear Models.

		Cultivar		Time[a]		Cultivar x Time	
	Year	**Deviance ratio** [b]	***P***	**Deviance ratio**	***P***	**Deviance ratio**	***P***
Recovery of *Plutella xylostella* caterpillars	2008	0.85 (3)	0.470	23.57 (5)	**< 0.001**[c]	0.92 (15)	0.547
	2009	0.34 (3)	0.799	35.31 (5)	**< 0.001**	4.51 (15)	**< 0.001**
Recovery of *Brevicoryne brassicae* aphids	2008[d]	1.96 (1)	0.166	2.69 (5)	**0.026**	1.73 (5)	0.137
	2009	0.39 (3)	0.763	18.35 (13)	**< 0.001**	1.60 (39)	**0.019**

[a] *The experiment was repeated over 6 weeks for P. xylostella in 2008 and 2009 and B. brassicae in 2008, and over 14 weeks for B. brassicae in 2009.* [b] *Deviance ratios were rounded off to two decimals. Between brackets the degrees of freedom.* [c] *Boldface values represent significant effects at P < 0.05.* [d] *For the recovery of B. brassicae in 2008, only the cultivars Christmas Drumhead and Badger were included, as too few aphids on Rivera and Lennox survived to allow for analysis of recovery*

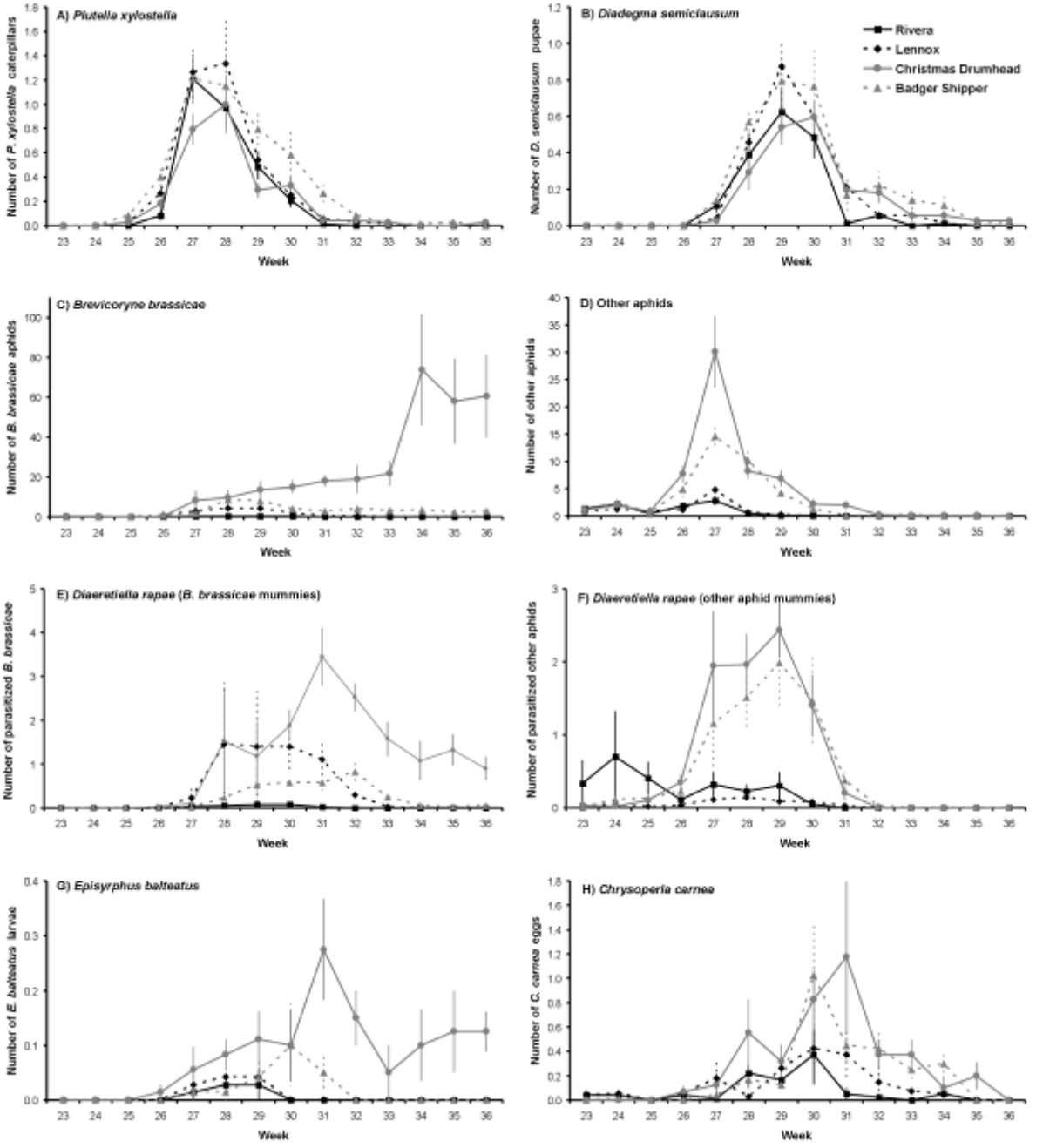
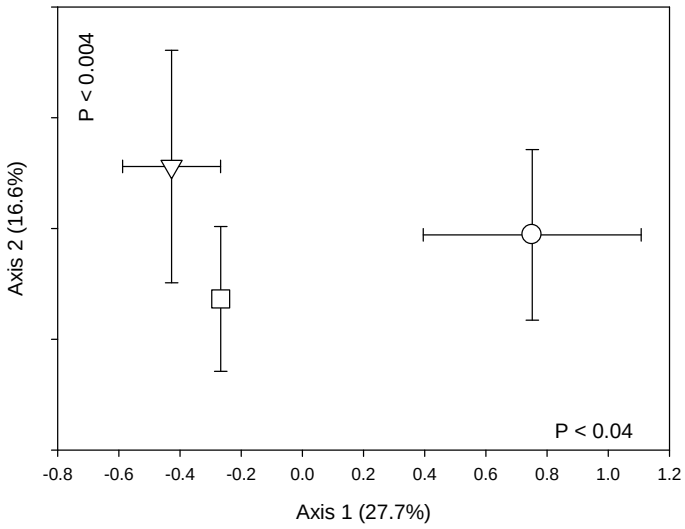

A) Plutella xylostella
Number of P. xylostella caterpillars
Week
B) Diadegma semiclausum
Number of D. semiclausum pupae
Rivera
Lennox
Christmas Drumhead
Badger Shipper
C) Brevicoryne brassicae
Number of B. brassicae aphids
D) Other aphids
Number of other aphids
E) Diaeretiella rapae (B. brassicae mummies)
Number of parasitized B. brassicae
F) Diaeretiella rapae (other aphid mummies)
Number of parasitized other aphids
G) Episyrphus balteatus
Number of E. balteatus larvae
H) Chrysoperla carnea
Number of C. carnea eggs

Fig. S4.1 *Insect abundance over time on plants of four Brassica oleracea cultivars in a common garden experiment in 2008 (A - B) and 2009 (C - H). (A) the lepidopteran herbivore Plutella xylostella (mean number of caterpillars per plant ± SE); (B) the parasitoid Diadegma semiclausum (mean number of pupae per plant ± SE); (C) the aphid Brevicoryne brassicae (mean number of aphids per plant ± SE); (D) other aphids (mean number of aphids per plant ± SE); (E) the aphid parasitoid Diaeretiella rapae (mean number of parasitized B. brassicae per plant ± SE); (F) the aphid parasitoid D. rapae (mean number of parasitized other aphids per plant ± SE); (G) the syrphid predator Episyrphus balteatus (mean number of larvae per plant ± SE); (H) the lacewing predator Chrysoperla. carnea (mean number of eggs per plant ± SE). For every data-point the sample size is 8, each sample consisting of the average number of insects recorded on nine plants in a plot.*

Table S5.1 Average values of several plant characteristics

	Badger Shipper			Rivera			*F*		
	Micro-organisms	**Control**	**Nematodes**	**Micro-organisms**	**Control**	**Nematodes**	**Cultivar**	**Treatment**	**Inter-action**
Biomass root sampling 1 (g)	1.55 ab	1.89 ab	1.29 ab	1.880 ab	2.19 a	1.23 b	0.76	4.34*	0.34
Biomass shoot sampling 1 (g)	6.61 a	7.51 a	7.03 a	6.34 a	6.45 a	6.27 a	2.91	0.53	0.33
Biomass root sampling 2 (g)	3.97 ab	4.27 b	4.01 ab	5.93 ab	6.34 a	5.69 ab	7.96*	0.33	0.20
Biomass shoot sampling 2 (g)	15.04 a	17.54 ab	16.23 a	19.41 ab	21.91 b	21.58 b	20.97*	1.89	0.18
RGR[a] (cm^2) first leaf, week 3-4	1.15 a	1.19 a	1.02 a	0.68 b	0.64 b	0.59 b	67.09*	3.10	0.45
RGR (cm^2) first leaf, week 4-5	2.37 a	2.13 a	2.20 a	1.99 a	2.15 a	1.98 a	4.49	0.36	1.60
RGR (cm^2) second leaf, week 3-4	0.56a	0.54 a	0.32 b	0.38 b	0.38 b	0.29 b	5.14*	3.04*	1.85
Total GLS[b] concentration leaf (µmol g^{-1} d.w.)	5.61 a	4.92 a	4.85 a	3.97 a	3.76 a	3.98 a	3.33	0.20	0.35
Total GLS concentration root (µmol g^{-1} d.w.)	12.52 a	13.73 a	9.10 a	16.42 a	8.95 a	10.85 a	0.02	1.98	1.90
Total GLS concentration phloem before aphid (nmol g^{-1} d.w.)	9.75 a	10.71 a	5.80 a	3.46 a	2.58 a	3.49 a	7.43*	0.23	0.46
Total GLS concentration phloem after aphid (nmol g^{-1} d.w.)	15.68 ab	4.20 a	6.65 ab	16.33 ab	16.32 b	17.87 ab	5.59*	3.42	1.06
Total phloem amino acid concentration before aphid (µmol g^{-1} d.w.)	13.84 a	11.40 a	8.04 a	9. 84 a	14.36 a	13.75 a	0.02	0.15	0.64
Total phloem amino acid concentration after aphid (µmol g^{-1} d.w.)	1.63 ac	0.68 a	1.31 ac	5.32 b	1.89 ac	5.02 bc	57.32*	10.42*	1.51

*[a] RGR = relative growth rate. [b] GLS = glucosinolate. * denote overall significant effect (two-way ANOVA: P<0.05). Means within a row followed by different letters are significantly different (Tukey post-hoc analysis: P<0.05)*



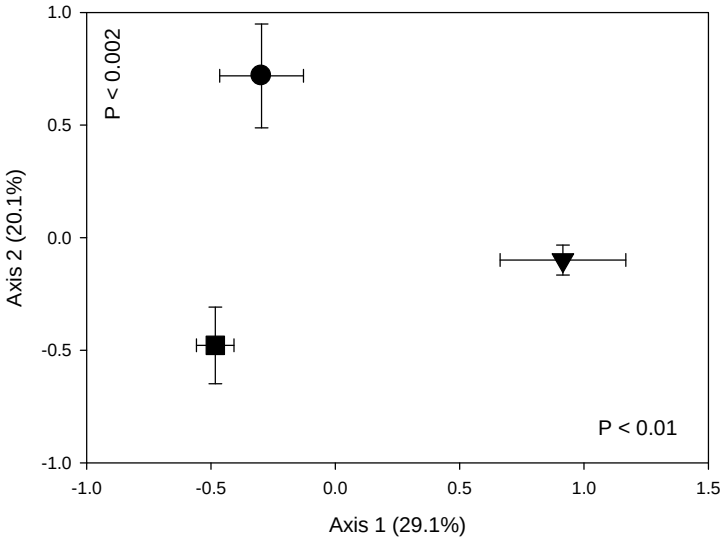
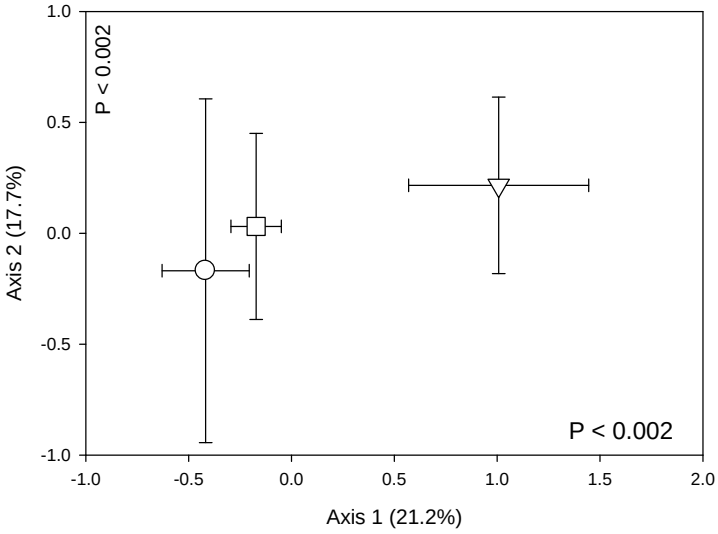
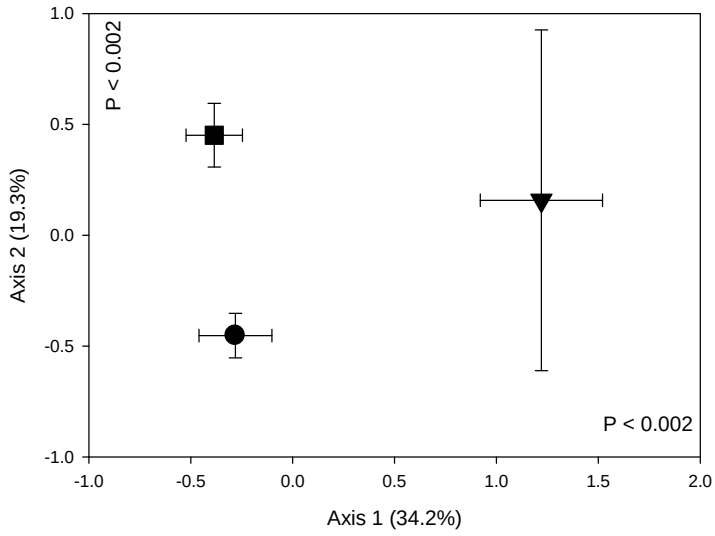


Fig. S5.1 *Correspondence analysis ordination diagram of denaturing gradient gel electrophoresis (DGGE) patterns of the bacterial (left page) and fungal (right page) communities. Points represent the mean of four samples with their 95% confidence interval. The P values on the first axis (lower right corner) and second axis (upper left corner) are determined by Monte Carlo permutation tests on the basis of distance-based redundancy analysis. This indicates that all treatments for both fungi and bacteria differ significantly from each other. White symbols represent samples from Rivera, black symbols represent Badger Shipper; circles: soils treated with micro-organisms, inverted triangles: untreated control soils, squares: nematode-treated soils.*

Rivera Fungal DGGE patterns



Badger Shipper Fungal DGGE patterns



Method S6.1 Generation of HAG1/MYB28 over-expression plants

Generation of HAG1/MYB28 over-expression plants was performed as described by Houshyani et al., in prep. The expression clone was kindly provided by Prof. Ulf-Ingo Flügge (University of Cologne, Germany). Briefly, the coding sequence of *HAG1/MYB28* (At5g61420.2) was amplified by RT-PCR and cloned into the pDONOR207 vector. LR reaction (Invitrogen Life Technologies) between pDONOR207 and pGWB2 recombined the insert from the entry clone into the destination vector. The binary plant transformation vector pGWB2 contained the CaMV 35S promotor and kanamycin and hygromycin resistance genes (Gigolashvili et al. 2007).

Subsequently, the expression clone was confirmed by digestion analysis (EcoRI + HindIII), gene-specific PCR primers and sequencing (Table below). A confirmed *Pro$_{35S}$:HAG1* clone was used to transfect *Agrobacterium tumefaciens* (strain Agl0) by electroporation and, after digestion and PCR confirmation, the *Pro$_{35S}$:HAG1* clone was transferred into *A. thaliana* accession Col-0 by flower dipping (Zhang et al. 2006). T1 seeds were harvested and transformed lines were selected on medium with kanamycin (50 µg ml^{-1}) and confirmed by kanamycin resistance gene (NptII) specific primers (Table next page). T2 generation seeds of one positive line (hereafter Col-0-MYB28) were harvested and used in the experiments. For selection of transformed seedlings and wild-type negative control plates, 30 µg ml^{-1} kanamycin was added to the growth medium.

Table. Primers used in this study

Gene Name	Sequence (5' to 3')
PCR analysis	
NptII	F: TGGGCACAACAGACAATCGGCTGC
NptII	R: TGCGAATCGGGAGCGGCGATACCG
MYB28	F: ACTGCGATGGACCAACTACC
MYB28	R: ACAACGATGATGGGGAGAAG
Sequencing	
pGWB2-MYB28_76	F: GAGCACGACACACTTGTCTA
pGWB2-MYB28_254	F: ATCATTGCGATAAAGGAAAG
pGWB2-MYB28_688	F: AGTTGTAGACTGCGATGGAC
pGWB2-MYB28_1005	F: CTCAATGCCTTTTCTGTCTC
pGWB2-MYB28_1395	F: CATGGACCAAGATTACGATT
pGWB2-MYB28_580	R: CTCCTTTCTTCAAGCCTTCT
pGWB2-MYB28_936	R: GTTGGAACTAGAAGCCAGTG
pGWB2-MYB28_1231	R: TAGCACTCAAGCTACCTTCC
pGWB2-MYB28_1623	R: CTTTTCAAGCGAGTCTGAGT
pGWB2-MYB28_1685	R: GAGCTCTAAGCGCTGTTATC

Method S6.2 Description of the GC-MS method and the identification of volatile compounds

GC-MS method

Prior to desorption of the volatiles, the samples were dry-purged under a flow of nitrogen at 20 ml min^{-1} for 20 min to remove moisture. The headspace samples were analysed using a Thermo Trace Gas Chromatography Ultra (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) quadrupole mass spectrometer (MS). The collected volatiles were released from the Tenax TA thermally on Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 240 ºC for 5 min under a helium flow of 30 ml min^{-1} while re-collecting the volatiles in an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK) at 5 ºC. Afterwards, the cold trap was rapidly heated at 40 °C s^{-1} to 260 °C and held for 7 min while the volatiles were transferred to a ZB-5MSi analytical column (30m x 0.25 mm I.D. x 1.0 µm film thickness; Phenomenex, Torrance, California, USA), in a splitless mode for further separation. The analytical column was set at initial temperature of 40 °C kept for 3.5 min and raised at 10 °C min^{-1} to 280 °C and held for 2.5 min under a column flow of 1 ml min^{-1} in a constant flow mode. The DSQ MS was operated in a scan mode with a mass range of 35 – 400 amu at 3.33 scans s^{-1} and ionization was performed in EI mode at 70 eV. MS transfer line and ion source were set at 275 and 250 °C, respectively.

Identification of compounds

Identification of compounds was based on comparison of mass spectra with those in the NIST 2005, Wiley and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) were also used as additional criterion for confirming the identity of the compounds. Relative quantification (peak areas of individual compounds) was performed using a single (target) ion, in selected ion monitoring (SIM) mode.

Table S6.1 Mean (± SE) chemical and morphological plant characteristics of uninfested plants of three *Arabidopsis thaliana* ecotypes and the transformed Col-0-MYB28 line

	***A. thaliana* ecotype**[a]			**Transformed *A. thaliana* line**[b]
Characteristic	**Cvi**	**Eri**	**Col-0**	**Col-0-MYB28**
Aliphatic GLS[c,d,e]	96.2 ± 5.2 **c**	36.7 ± 1.7 **b**	15.3 ± 1.2 **a**	31.3 ± 2.6 *****
Indole GLS[c,d]	3.4 ± 0.2 **a**	7.2 ± 0.4 **c**	4.6 ± 0.4 **b**	4.4 ± 0.3 **ns**
Total GLS[d,e]	99.6 ± 5.3 **c**	43.9 ± 2.0 **b**	20.3 ± 1.4 **a**	35.7 ± 2.8 *****
Biomass (mg)	642.4 ± 32.6 **a**	790.5 ± 32.9 **b**	549.5 ± 27.8 **a**	635.4 ± 47.1 **ns**
Trichome density[e,f]	60.7 ± 3.3 **c**	17.5 ± 1.3 **a**	26.3 ± 1.0 **b**	25.8 ± 2.0 **ns**

*n = 10. [a] Different letters denote differences in means among the three ecotypes as analysed by ANOVA and post-hoc Tukey tests. [b] * denotes significant difference (P<0.05) and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analysed by Student's t-tests. [c] Glucosinolates (GLS) are grouped according to their biosynthetic origin. [d] µmol g^{-1} dry weight leaf. [e] Variable was log-transformed in statistical analysis to obtain normality. [f] Trichome density is the number of trichomes per 25 mm^2 leaf area of the 6^{th} or 7^{th} youngest leaf.*

Table S6.2 Trivial and scientific names of the glucosinolate (GLS) compounds

Abbreviation	Trivial name	Scientific name
Aliphatic GLS		
ALY	glucoalyssin	5-methylsulfinylpentylGLS
EPRO	epiprogoitrin	2-(S)-2-hydroxy-butenylGLS
ERU	glucoerucin	4-methylthiobutylGLS
GNA	gluconapin	3-butenylGLS
GBN	glucobrassicanapin	4-pentenylGLS
GNL	gluconapoleiferin	2-hydroxy-4-pentenylGLS
HIR	glucohirsutin	8-methylsulfinyloctylGLS
IBE	glucoiberin	3-methylsulfinylpropylGLS
IBV	glucoiberverin	3-methylthiopropylGLS
RAPH	glucoraphanin	4-methylsulfinylbutylGLS
SBE	glucosiberin	7-methylsulfinylheptylGLS
SIN	sinigrin	2-propenylGLS
3OH	no trivial name	3-hydroxypropylGLS
7THIO	no trivial name	7-methylthioheptylGLS
Indole GLS		
GBC	glucobrassicin	3-indolylmethylGLS
NEO	neo-glucobrassicin	1-methoxy-3-indolylmethylGLS
4MeOH	4-methoxyglucobrassicin	4-methoxy-3-indolylmethylGLS
4OH	4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethylGLS

Table S6.3 Volatile compounds detected in the headspace of aphid-infested *Arabidopsis thaliana* plants

No	Compound	CAS no	[a]RT (min)	[b]$LRI_{exp.}$	[c]$LRI_{lit.}$	[d]Target Ion	[e]Ident.
1	3-butene nitrile	109-75-1	5.51	657	658	67	MS, LRI
2	2-pentanone	107-87-9	6.17	685	687	86	MS, LRI
3	4-methyl-2-pentanone	107-87-9	7.45	741	735	58	MS, LRI
4	3-methyl-3-butene nitrile	4786-19-0	7.98	763	NA	81	MS
5	1-pentanol	71-41-0	8.05	766	766	55	MS
6	2-hexanone	591-78-6	8.58	789	788	58	MS, LRI
7	butyl acetate	123-86-4	9.12	814	813	61	MS, LRI
8	2-pentyl acetate	626-38-0	9.87	849	829*	87	MS, LRI
9	styrene	100-42-5	10.90	898	897	104	MS, LRI
10	cumene	98-82-8	11.58	934	934	120	MS, LRI
11	isocumene	103-65-1	12.15	964	964	120	MS, LRI
12	3-butenyl isothiocyanate	3386-97-8	12.66	991	978	113	MS, LRI
13	hemimellitene	526-73-8	12.92	1005	1002	120	MS, LRI
14	p-cymene	99-87-6	13.46	1036	1034	134	MS, LRI
15	limonene	138-86-3	13.57	1042	1039	136	MS, LRI
16	o-cresol	95-48-7	13.82	1056	1054	108	MS, LRI
17	m-cymene	535-77-3	14.05	1070	1082	119	MS, LRI
18	ɣ-terpinene	99-85-4	14.06	1070	1074	93	MS, LRI
19	linalool	78-70-6	14.64	1104	1103	93	MS, LRI
20	cis-limonene-1,2-epoxide	1195-92-2	15.38	1150	1139*	119	MS, LRI
21	menthol	1490-04-6	16.02	1189	1185	123	MS, LRI
22	1-methylene-1H-indene	2471-84-3	16.41	1215	NA	128	MS
23	methyl salicylate	119-36-9	16.42	1215	1208	92	MS, LRI
24	diethyl-2-methylene succinate	2409-52-1	16.63	1229	1218	113	MS, LRI
25	cyclosativene	22469-52-9	19.17	1406	1378	204	MS, LRI
26	daucene	16661-00-0	19.24	1411	1382	204	MS, LRI
27	ɣ-elemene	339154-91-5	19.74	1449	1433	121	MS, LRI
28	longifolene	475-20-7	19.82	1455	1448	161	MS, LRI
29	δ-selinene	28264-28-4	20.59	1514	1493	204	MS, LRI
30	6-methyl-α-ionone	79-69-6	20.97	1544	1518	93	MS, LRI
31	lilial	80-54-6	21.08	1553	1535	189	MS, LRI
32	farnesylacetaldehyde	66408-55-7	24.58	1857	1861	69	MS, LRI

[a]RT: Retention time of compounds in the chromatographic window. [b]LRI_{exp}: linear retention indices experimentally obtained. [c]LRI_{lit}: linear retention indices obtained from literature [NIST 2005, Wageningen University Mass Spectral library, and The Pherobase (http://www.pherobase.com/database/kovats/kovats-index.php) on a column with (5%-Phenyl)-methylpolysiloxane stationary phase or equivalent. [d] Target Ion used for relative quantitation to obtain the peak areas of each corresponding compound. [e] Identification (Tent.) based on retention indices (RI) and/or mass spectra (MS). [*] LRI_{lit}: LRI on a 100% polydimethylsiloxane (PDMS) or equivalent stationary phase. NA: Not Available.

Table S7.1 Artificial diet for *Spodoptera exigua*

Ingredients
5 L water
140 g agar
700 g corn flour
250 g brewer's yeast
250 g wheat germ
10 g sorbic acid
8 g nipagin (methyl, 4 hydroxybenzoate)
40 g ascorbic acid (vitamin C)
0.5 g streptomycin sulphate

Method S8.1 Generation of transgenic *FaNES1*-expressing plants

Phusion enzyme (Finnzymes, Finland) was used for PCR when necessary. Standard cloning methods (restriction and ligation) were used for construction of all the plasmids and the vectors of final and intermediate stages and were checked by restriction analysis and sequencing.

The cDNA fragments encoding amino acid residues of the AtFPS2 (At4g17190) and FaNES1 proteins were cloned into two separate pGEM-T plasmid (Promega) (Cunillera et al. 1996; Kappers et al. 2005). The BamHI/ NotI restricted fragment of resulted clones containing the FPS2 and FaNES1 coding sequences were gel purified and ligated in the pIV2B_2.5 and pIV2A_2.5 entry vectors, respectively, containing a CaMV 35S promoter, CoxIV mitochondrial targeting sequence and a RbcS1 terminator (www.pri.wur.nl/UK/products/ImpactVector/). Resulting entry clones were transferred to *E. coli* strain X1-Blue by heat shock for propagation. LR reaction (Invitrogen Life Technologies) was performed to recombine the sequences of interest in the entry clones between the right and left borders of the T-DNA in the pBINPLUS binary vector (van Engelen et al. 1995). This resulted in the 2way plasmid contained the AtFPS2 and FaNES1 coding sequence each delimited by CaMV 35S promoter, CoxIV mitochondrial targeting sequence and a RbcS1 terminator. This binary vector was introduced to X1-Blue by heat shock and to *Agrobacterium tumefaciens* strain AgII by electroporation.

Plant transformations were performed using the *Arabidopsis* flower dipping method (Zhang et al. 2006). T1 seeds were harvested and T1 transgenic plants were selected on kanamycin plates (purified agar 0.8% + 2.2 g L^{-1} 0.5 MS + vitamins; pH 6; 50 µg ml^{-1} kanamycin) and confirmed by kanamycin resistance gene (NptII) specific primers. T2 generation seedlings of positive lines were used in the experiments.

Table S8.1 Volatile compounds detected in the headspace of aphid-infested wild-type and transgenic *FaNES1*-expressing *Arabidopsis thaliana* plants

No	Compound	CAS no	[a]RT (min)	[b]$LRI_{exp.}$	[c]$LRI_{lit.}$	[d]Target Ion	[e]Ident.
1	3-butene nitrile	109-75-1	5.51	657	658	67	MS, LRI
2	1-methoxy-2-propanol	107-98-2	5.89	673	672	47	MS, LRI
3	1-penten-3-ol	616-25-1	6.09	682	682	57	MS, LRI
4	2-pentanone	107-87-9	6.19	686	687	86	MS, LRI
5	acetoin	513-86-0	6.7	708	705	45	MS, LRI
6	methyl cyclohexane	108-87-2	7.15	728	726	83	MS, LRI
7	dimethyl disulfide	624-92-0	7.65	749	752	94	MS, LRI
8	1-pentanol	71-41-0	8.05	766	766	42	MS, LRI
9	2,2-dimethyl propanoic acid	75-98-9	8.73	796	790	102	MS, LRI
10	(*E*)-2-hexenal	6728-26-3	9.98	855	854	98	MS, LRI
11	2-Methylallyl acetate	820-71-3	10.01	856	NA	72	MS
12	(*Z*)-Hex-3-en-1-ol	928-96-1	10.04	857	859	67	MS, LRI
13	1-methoxy-2-propyl acetate	108-65-6	10.24	867	857*	72	MS, LRI
14	allyl isothiocyanate	57-06-7	10.67	887	887	99	MS, LRI
15	1-nonene	124-11-8	10.76	892	892	56	MS, LRI
16	anisole	100-66-3	11.4	924	918	108	MS, LRI
17	α-pinene	80-56-8	11.81	946	940	93	MS, LRI
18	5-ethyl-2(5H)-furanone	80-56-9	12.03	957	984*	112	MS, LRI
19	propylbenzene	103-65-1	12.15	964	964	91	MS, LRI
20	3-butenyl isothiocyanate	3386-97-8	12.64	990	978	113	MS, LRI
21	mesitylene	108-67-8	12.66	991	995	105	MS, LRI
22	hemimellitene	526-73-8	12.91	1004	1007	105	MS, LRI
23	(*Z*)-4-hexen-1-ol, acetate	42125-17-7	12.93	1005	1005	67	MS, LRI
24	2,2'-dihydroxydipropyl ether	110-98-5	13.2	1021	1003	89	MS, LRI
25	pseudocumene	95-63-6	13.49	1034	1026	105	MS, LRI
26	2,2'-oxybis-1-propanol	108-61-2	13.53	1040	1051	59	MS, LRI
27	limonene	138-86-3	13.55	1041	1039	68	MS, LRI
28	(*E*)-β-ocimene	3779-61-1	13.74	1052	1044	93	MS, LRI

29	indane	496-11-7	13.78	1054	1048	117	MS, LRI
30	phenylacetaldehyde	122-78-1	13.8	1055	1055	120	MS, LRI
31	linalool	78-70-6	14.63	1103	1103	121	MS, LRI
32	(*E*)-DMNT (4,8-dimethylnona-l,3,7-triene)	19945-61-0	14.9	1120	1113	150	MS, LRI
33	3-acetyl-2,5-dimethylfuran	10599-70-9	14.92	1121	1103	138	MS, LRI
34	4-methyl-2-undecene	91695-32-8	15.11	1133	1158	69	MS, LRI
35	(*S*)-(+)-6-methyl-1-octanol	110453-78-6	15.3	1145	NA	97	MS
36	1-methylene-1H-Indene	2471-84-3	16.41	1215	NA	128	MS
37	methyl salicylate	119-36-9	16.41	1215	1208	92	MS, LRI
38	2,4-dimethyl benzalde-hyde	15764-16-6	16.75	1237	1180	134	MS, LRI
39	6-ethyltetralin	22531-20-0	18.61	1366	1340*	131	MS, LRI
40	clovene	469-92-1	19.13	1403	1396	161	MS, LRI
41	longicyclene	1137-12-8	19.31	1417	1382	204	MS, LRI
42	isolongifolene	1135-66-6	19.56	1435	1402	148	MS, LRI
43	β-caryophyllene	87-44-5	19.7	1446	1440	133	MS, LRI
44	allo-isolongifolene	87064-18-4	19.74	1449	1412	95	MS, LRI
45	longifolene	475-20-7	19.81	1454	1448	161	MS, LRI
46	α-thujaplicinol	16643-33-7	20.21	1484	1509	165	MS, LRI
47	α-neoclovene	4545-68-0	20.44	1502	1497	189	MS, LRI
48	(*E,E*)-α-farnesene	502-61-4	20.62	1516	1520	93	MS, LRI
49	(*E*)-nerolidol	40716-66-3	21.37	1577	1571	93	MS, LRI
50	(*E,E*)-TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene)	62235-06-7	21.52	1589	1565	69	MS, LRI
51	hexyl salicylate	6259-76-3	22.89	1704	1682	222	MS, LRI
52	2-ethylhexyl salicylate	118-60-5	24.34	1834	1807	120	MS, LRI
53	farnesylacetaldehyde	66408-55-7	24.57	1856	1861	69	MS, LRI

*[a]RT: Retention time of compounds in the chromatographic window. [b]LRI_{exp}: linear retention indices experimentally obtained. [c]LRI_{lit}: linear retention indices obtained from literature [NIST 2005, Wageningen University Mass Spectral library, and The Pherobase (http://www.pherobase.com/database/kovats/kovats-index.php) on a column with (5%-Phenyl)-methylpolysiloxane stationary phase or equivalent. [d] Target Ion used for relative quantitation to obtain the peak areas of each corresponding compound. [e] Identification (Tent.) based on retention indices (RI) and/or mass spectra (MS). * LRI_{lit}: LRI on a 100% polydimethylsiloxane (PDMS) or equivalent stationary phase. NA: Not Available.*

List 9.1 Step-wise question list to facilitate the use by regulators

If the answer to any of the following questions is 'no', this may indicate a lack of rigour in the set-up of the study that was performed to test the ecological non-target effects of the transgenic crop. If the answer to all questions is 'yes', the study was performed properly.

1) Did the applicant provide data representing the baseline variation?

- Was a range of genotypes/varieties selected for representation of the baseline variation?
- Were the selected genotypes/varieties of the same (or a closely related) species?
- Were several environmental conditions (*e.g.* soil types and climates) used for baseline representation?
- Was the selection of varieties/environments based on a relevant trait (for example metabolomics profile or resistance to herbivores)?

2) Did the applicant measure the traits under representative testing conditions?

- Was a tiered (stepwise) approach used (see Fig. 2.2 in **Chapter 2**)?
- Were field studies performed?
- Were multiple environmental conditions (*e.g.* soil types and climates) selected for field studies?
- Were the selected environmental conditions relevant for the crop species, *i.e.* based on common cultivation practice?

3) Did the applicant test relevant organisms and traits?

- Were relevant non-target organisms selected? (see Table 9.1)
- Were both aboveground and belowground organisms included?
- Was fitness of the non-target organisms quantified, *i.e.* by using several performance parameters as proxies?
- Do the selected performance parameters have the ability to demonstrate both lethal and sub-lethal effects?
- Were the non-target effects studied during multiple generations (*e.g.* during multiple field seasons)?
- Was behaviour of the non-target organisms studied?

4) Did the applicant interpret the results correctly?

- Was the sample size large enough to prevent type I and II errors and was pseudoreplication prevented? (see Charleston and Dicke 2008)
- Was the null hypothesis correctly formulated as: 'the effects of the transgenic crop are not significantly different from the full complement of effects found in the baseline variation'?
- Were relevant statistical methods used and were the data interpreted correctly to accept (*i.e.* a failure to reject) the null hypothesis?

The research described in this thesis was financially supported by a grant from the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW) under the ERGO program (grant number 838.06.010).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Lay-out by author.
Cover design by Hans Smid.
Cover photographs by Hans Smid, Nina Fatouros (www.bugsinthepicture.com) and Martine Kos.
Photographs page 10, 26, 184 by Nina Fatouros and photograph page 198 by Tibor Bukovinszky (www.bugsinthepicture.com).

Printed by: Wöhrmann Print Service, Zutphen, The Netherlands.