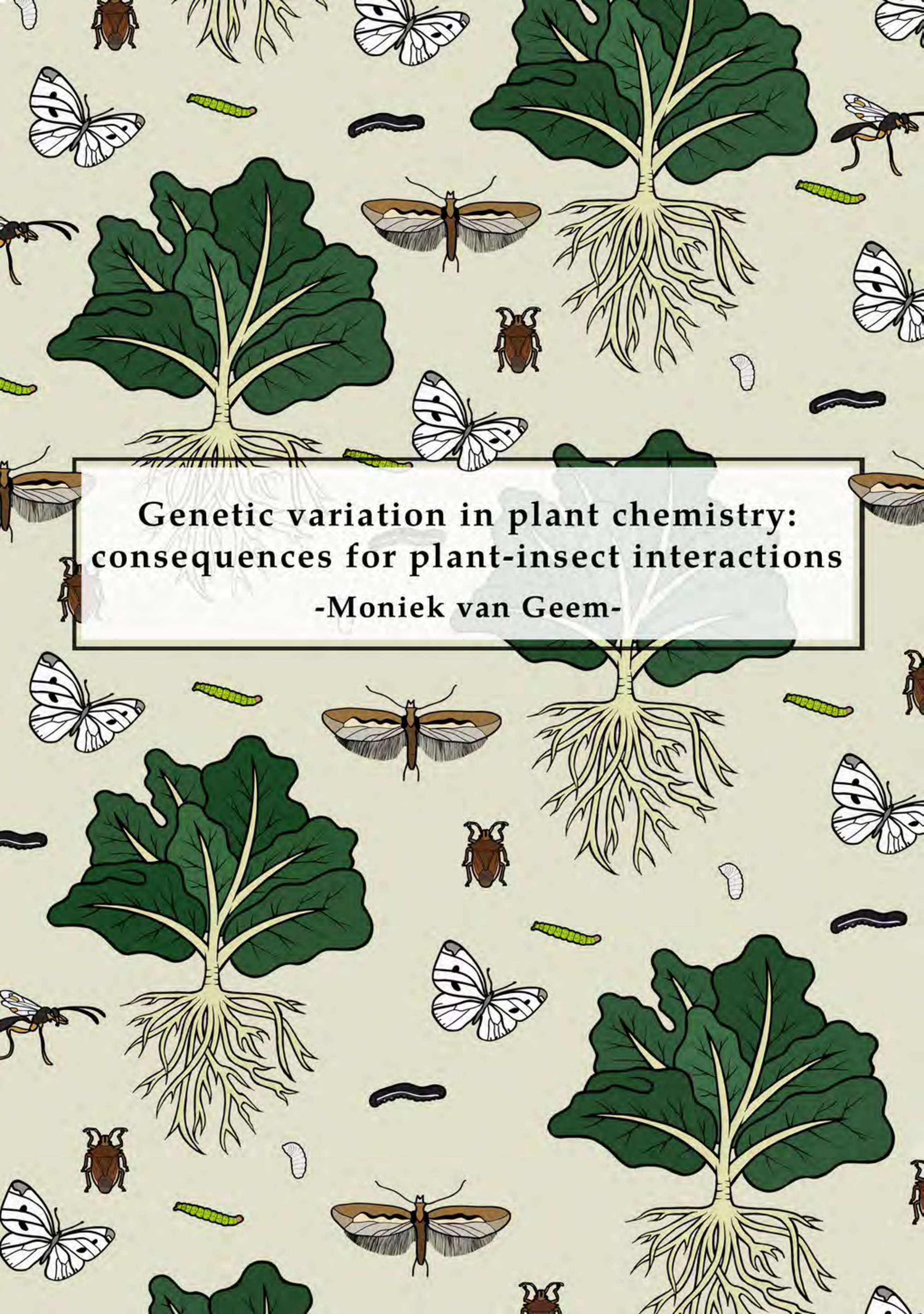

Genetic variation in plant chemistry: consequences for plant-insect interactions

-Moniek van Geem-

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consequences for plant-insect interactions

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This research was conducted under the auspices of the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC)

Genetic variation in plant chemistry:

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 20 April 2016
at 4 p.m. in the Aula.

Moniek van Geem
Genetic variation in plant chemistry: consequences for plant-insect interactions,
142 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summaries in Dutch and English

ISBN 978-94-6257-668-1

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Chapter 1

General introduction

Plants are unique organisms in the sense that they reside in both soil and air at the same time and need input from both compartments in order to survive, grow and reproduce. They form the basis of many food webs and are consumed by a wide variety of organisms, ranging from large vertebrate ungulates to microscopic invertebrate nematodes. Insects are the most diverse group of animals on this planet, inhabiting a wide range of habitats and displaying huge variation in body shapes and feeding modes, with approximately 50% of the species being phytophagous (Price et al. 2011). Plants are thus under attack by a large and phylogenetically and functionally diverse group of herbivorous insects.

Plant defence

There are various ways in which plants can defend themselves against herbivore attacks. Two defence tactics can be distinguished: tolerance and resistance (van der Meijden et al. 1988; Karban and Baldwin 1997), which are not necessarily mutually exclusive. Tolerance concerns the use of resources for regrowth and reproduction after tissue damage (Strauss and Agrawal 1999). Resistance involves the use of traits to reduce or prevent attack by antagonists, affecting their feeding preference or performance (Karban and Baldwin 1997; Strauss and Agrawal 1999). Some of these defence traits are morphological (e.g. trichomes or waxy leaf surfaces to impede movement) while others are chemical (e.g. secondary metabolites) (Bezemer and van Dam 2005). Throughout this thesis I will use the term 'defence' while referring to resistance for simplicity, as is common in literature on plant-insect interactions.

Primary metabolites such as amino acids and carbohydrates are produced by plants and are essential for their growth, development and reproduction. Secondary metabolites are not essential for plant survival, but they are part of the chemical defence system of plants (Hartmann 1996). Defensive secondary metabolites (a.k.a. allelochemicals) can repel attacking herbivores or can negatively influence their development, thus reducing the quality of the plant as a food source (Schoonhoven et al. 2005). Secondary metabolites may already be present in plant tissues (i.e. constitutive defence) or increase/are only expressed after tissue damage (i.e. induced defence) (Karban and Baldwin 1997; van Dam 2009). The expression of secondary metabolites may be restricted to the damage site (i.e. local) or may also be expressed in other parts of the plant (i.e. systemic) (Bezemer and van Dam 2005, Karban and Baldwin 1997).

Plant - insect interactions

Plants and specialist herbivorous insects are often involved in a co-evolutionary arms race in which plants evolve mechanisms to repel or negatively affect the performance of attacking insects, whereas insects evolve counter-mechanisms to overcome these defence strategies (Ehrlich and Raven 1964). Generalist herbivorous insects are generally less well adapted to the defences of plants than specialists (Krieger et al. 1971; Ali and Agrawal 2012; Barrett and Heil 2012). Specialist herbivorous insects have several ways to overcome the chemical defence of a plant, such as detoxification, excretion and sequestration of plant allelochemicals (Nishida 2002; Renwick 2002; Despres et al. 2007; Opitz and Muller 2009; Winde and Wittstock 2011).

Plant quality for herbivores is not only determined by secondary metabolites but also by primary metabolites (Scriber and Slansky 1981; Awmack and Leather 2002), and more specifically by their interaction (Slansky 1986). Plants produce primary metabolites, such as amino acids and carbohydrates, for their growth, development and reproduction. For insects, these primary metabolites provide nutrients that are also essential for their development. When insects feed on plants, this herbivory usually affects both primary and secondary plant chemistry (Gange and Brown 1989; Johnson et al. 2009). Plants can reallocate primary metabolites to other plant organs in response to herbivory, which affects the nutritional quality of the plant (Masters and Brown 1992; Masters et al. 1993).

Since the publication of the seminal paper by Price et al. (1980), the focus of plant-insect studies has shifted from bi-trophic interactions (i.e. plants and herbivores) to multi-trophic interactions where higher trophic levels, such as natural enemies, are also included. Defence chemistry of plants does not only affect attacking herbivorous insects, but also their natural enemies. The indirect effect of plant chemistry –via the herbivore– on the performance of natural enemies has been well studied (Barbosa et al. 1991; Harvey et al. 2003; Harvey 2005; Ode 2006; van Dam and Heil 2011; Johnson et al. 2013), showing that plant quality can indirectly affect the performance of the third and even fourth trophic level.

Aboveground - belowground interactions

Plants are not only attacked aboveground (stems, leaves, flowers and seeds), but also belowground (roots). A wide variety of taxonomic groups and feeding modes exists belowground, such as micro-arthropods, protozoa, bacteria, nematodes and fungi (Brown and Gange 1990; Killham 1994). Some organisms are generally beneficial to the plant (e.g. nitrogen fixing bacteria), whereas others are antagonistic (e.g. root herbivores) (Wardle et al. 2004; Raaijmakers et al. 2009; van Dam 2009).

Having structures in both the air and the soil, terrestrial plants mediate interactions between organisms in the aboveground and belowground compartments. Herbivores feeding in one compartment can affect plant traits, such as those associated with defence, in the other compartment when plants exhibit a systemic response to herbivore attack. Herbivory may also cause changes in the primary metabolite profiles of plant structures. The fact that the above- and belowground compartments are linked through plants has received increasingly more attention in the last two decades (Bardgett et al. 1998; Hooper et al. 2000; van der Putten et al. 2001; Blossey and Hunt-Joshi 2003; Wardle et al. 2004; van Dam and Heil 2011; Johnson et al. 2012; Soler et al. 2012). One of the pivotal questions in these studies is how aboveground-belowground plant defence is organized. Most of these studies have focussed on interactions between aboveground and belowground herbivores and natural enemies (e.g. Soler et al. 2007a). However, relatively few studies examined how plant intra-specific genetic variation may influence plant defences (but see Gols et al. 2008b; van Leur et al. 2008).

Genetic variation in plant defence chemistry

Natural plant populations often display genetic variation in the expression of various phenotypic traits such as morphology, phenology and both primary and secondary chemistry. Plant secondary chemistry is subject to genetic variation at different scales (Hartmann 1996; Hoy et al. 1998; Agrawal et al. 2012). Variation exists between plant families: for instance, alkaloids are generally found in Solaneceous species and glucosinolates generally in Brassicaceous species (Bennett and Wallsgrove 1994). Other studies have shown that the total concentrations and concentrations of specific compounds of secondary metabolites differ between species within a plant family (Fahey et al. 2001), between populations of one species (Arany et al. 2008; Gols et al. 2008b), and between individuals within a population (Fahey et al. 2001). For example, foliar concentrations of sideroxylonal, a defence compound of eucalyptus trees, differ between trees on a small spatial scale (Andrew et al. 2007). Defence chemistry also may differ between different organs of an individual plant (e.g. seeds, leaves and roots) (Häring et al. 2007) and different ontogenetic stages of a plant (Kearsley and Whitham 1989; Boege and Marquis 2005). As a consequence of the large variation in plant chemistry, herbivores may encounter a wide range of plant metabolites, depending on the species, population, individual and even organ of the plant they are feeding on.

Genetic variation in heritable plant traits that influence their fitness is a prerequisite for natural selection and evolution to take place (Whitham et al. 2003; Hughes et al. 2008). Genetic variation in plant (defence) traits can be under selection pressure from different biotic and abiotic factors (Hartmann 1996; Agrawal 2004; Lankau 2007; Arany et al. 2009). These selection pressures vary both in time and space, creating specific suits of (a)biotic factors affecting plant traits. Above-belowground interactions may impose strong selection pressures on plant defence chemistry. Vice versa, genetic variation in plant defence chemistry can have important consequences for above-belowground interactions.

As a consequence of genetic variation, plants will respond differently to insect herbivory. The total concentrations of primary and secondary metabolites and the concentrations of individual compounds may change differentially in response to herbivory. This in turn influences the effect that plant chemistry has on a herbivore, since herbivores are differentially affected by total concentrations and individual compounds. So far, there have been very few studies that examined the effect of naturally occurring genetic variation in plant chemistry on (multi)trophic plant-insect interactions in an above-belowground system framework (but see van Leur et al. 2006; 2008). The main aim of this thesis is to study the effect of genetic variation in plant chemistry on above- and belowground insect interactions.

Model system

Wild cabbage plants (*Brassicae oleracea* L.) are perennials that can grow as old as twenty years (Mitchell and Richards 1979). Natural populations grow on chalk soil along the Atlantic coasts of France, Ireland, the UK and Denmark. Older plants become large and sturdy, with woody stems and thick leaves. They can grow on flat terrain but also on steep slopes and even on vertical cliff edges. *Brassica oleracea* is commonly believed to be the ancestor of a wide variety of cultivars that include cauliflower, broccoli, Brussels sprouts, white cabbage and kohlrabi.

This thesis focuses on wild cabbage populations that grow along the Dorset coast of the UK, near Swanage (chapter 4, Fig. 1). We selected five populations that are located within close proximity of each other but are known to genetically differ in their defence chemistry profiles (Mithen et al. 1995, Moyes et al. 2000; Gols et al. 2008b). Wild cabbage belongs to the Brassicaceae, a plant family that contains more than 300 genera and is characterized by the production of glucosinolates, a group of secondary metabolites (Fahey et al. 2001).

The defensive action of glucosinolates is known as the glucosinolate-myrosinase system (Bones and Rossiter 1996). Glucosinolates and the enzyme myrosinase are stored separately in plant tissues but can come into contact with each other after tissue damage, for example when insects are feeding on the plant. When glucosinolates come into contact with myrosinase, they are hydrolysed into breakdown products like nitriles and isothiocyanates (Halkier and Gershenzon 2006; Winde and Wittstock 2011). Glucosinolates and their breakdown products can act as fungicidal, bactericidal, nematicidal and allelopathic compounds (Fahey et al. 2001; Halkier and Gershenzon 2006).

Three classes of glucosinolates can be distinguished based on the origin of the amino acid side chain that serves as a precursor for glucosinolates biosynthesis: aliphatic, aromatic and indole glucosinolates (Halkier and Gershenzon 2006). Aliphatic glucosinolates in wild cabbage have been shown to affect the performance of generalist herbivorous insects (Gols et al. 2008b), whereas specialist herbivorous insects seem to be more affected by indole glucosinolates (Gols et al. 2008b; Harvey et al. 2011). Plants from the Winspit population exhibit relatively high concentrations of aliphatic and total glucosinolates, whereas plants from the Kimmeridge population exhibit relatively high concentrations of indole glucosinolates (Gols et al. 2008b; Harvey et al. 2011; Abdalsamee and Muller 2012).

Research objectives and thesis outline

The main aim of my thesis is to study how genetic variation in plant chemistry affects (multi)trophic interactions between plants and associated insects, both above- and belowground. I explore this topic with wild *B. oleracea* plants, using a variety of herbivorous insects with or without higher trophic levels, examining (multi)trophic interactions in the above-or belowground compartment, or both at the same time.

In **chapter 2** I review the role of aboveground-belowground interactions in the evolution and maintenance of genetic variation in plant defence chemistry. I review literature on this topic and identify gaps in our knowledge about what drives genetic variation in plant traits related to above- and belowground (multi)trophic plant-insect interactions.

To overcome the chemical defence of plants, herbivorous insects have developed several adaptations to overcome those defence. One adaptation is the sequestration of plant secondary metabolites from food plants, which can be used by insects for their own defence. In **chapter 3** I study whether sequestration of glucosinolates by a specialist herbivore is an effective defence against a generalist predator. I compared the performance of a generalist predatory bug, the spined soldier bug (*Podisus maculiventris*), when feeding on a sequestering herbivore (*Athalia rosae*) and on a non-sequestering herbivore (*Pieris rapae*) that had been reared on one of three different wild cabbage populations.

The effect of genetic variation in glucosinolate concentrations has been well studied for aboveground plant-insect interactions, but not for belowground interactions. In **chapter 4** I compare the performance of a specialist belowground herbivore, the cabbage root fly (*Delia radicum*), on five wild cabbage populations differing in their defence chemistry. I measured the primary (amino acids and sugars) and secondary (glucosinolates) chemistry in the roots of plant that had been exposed to root herbivory, compared that to the root chemistry of plants that had not experienced root herbivory, and statistically analysed the data for correlations between herbivore performance and root chemistry.

In **chapter 5** I investigate the effect of belowground herbivory on an aboveground trophic chain on three wild cabbage populations. I inoculated plants belowground with larvae of the cabbage root fly, *D. radicum*. For the aboveground trophic chain, I used the diamondback moth *Plutella xylostella*, a specialist herbivore on crucifers, and its natural enemy, the endoparasitic wasp *Cotesia vestalis*. Besides recording insect performance, I also measured primary and secondary chemistry in both the leaves and the roots and statistically analysed the data for correlations between herbivore performance and plant chemistry.

In the final chapter, **chapter 6**, I discuss and synthesize the main findings of this thesis. I conclude that the effect of genetic variation in plant (defence) chemistry on herbivorous insects (and their natural enemies) is species dependent and often interacts with other factors such as the presence of a herbivore in the opposite compartment. Including genetic variation into (multi)trophic above-belowground interactions adds more complexity but also more closely resembles natural conditions.

Chapter 2

The importance of aboveground-belowground interactions on the evolution and maintenance of variation in plant defence traits

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Published in *Frontiers in Plant Science* 4 (2013)

Abstract

Over the past two decades a growing body of empirical research has shown that many ecological processes are mediated by a complex array of indirect interactions occurring between rhizosphere-inhabiting organisms and those found on aboveground plant parts. Aboveground - belowground studies have thus far focused on elucidating processes and underlying mechanisms that mediate the behaviour and performance of invertebrates in opposite ecosystem compartments. Less is known about genetic variation in plant traits such as defence as that may be driven by above- and belowground trophic interactions. For instance, although our understanding of genetic variation in aboveground plant traits and its effects on community-level interactions is well developed, little is known about the importance of above-below ground interactions in driving this variation. Plant traits may have evolved in response to selection pressures from above- and belowground interactions from antagonists and mutualists. Here, we discuss gaps in our understanding of genetic variation in plant- related traits as they relate to aboveground and belowground multi-trophic interactions. When metabolic resources are limiting, multiple attack by antagonists in both domains may lead to trade-offs. In nature, these trade-offs may critically depend upon their effects on plant fitness. Natural enemies of herbivores may also influence selection for different traits via top-down control. At larger scales these interactions may generate evolutionary 'hotspots' where the expression of various plant traits is the result of strong reciprocal selection via direct and indirect interactions. The role of abiotic factors in driving genetic variation in plant traits is also discussed.

Introduction

Of the many traits possessed by plants that are closely tied with their growth, survival and fitness, those relating to defence have been especially well studied over many years (see reviews by Karban and Baldwin 1997 and Schoonhoven et al. 2005). Defences in plants are often divided into direct and indirect defences. Direct defences are aimed directly at the attackers, such as herbivores, and include morphological (e.g. trichomes or sticky glands) and chemical (toxic secondary compounds) traits that interfere with colonisation, feeding and development of the herbivore. For example, toxic secondary compounds can act as feeding deterrents or negatively alter the performance of a herbivore through increased mortality, slower growth rates or reduced fitness (Schoonhoven et al. 2005). Indirect defences are aimed at promoting the efficiency of natural enemies, such as predators or parasitic wasps (parasitoids) that kill the herbivores and thus reduce their damage to the plant. Indirect defences may also be morphological (e.g. domatia) or chemical (e.g. the production of attractive volatiles, energy sources). Both direct and indirect defences are expressed constitutively in many plants, meaning that they are always expressed whereas in others they are often inducible, meaning that initial levels are low but increase after attack (Karban and Baldwin 1997). These traits are often species- (or even genotype) specific, and are assumed to depend on the predictability of attacks from antagonists and susceptibility of plants to these attacks.

Unlike most terrestrial biota, the vast majority of plants occupy two connected ‘compartments’ - the open air and soil - that differ in many biotic and abiotic properties. Above-ground plant structures include stems, branches, leaves, shoots, flowers and seeds, whereas the soil is dominated by the root system. These differing plant structures facilitate interactions between biotic communities that rarely come into direct physical contact with one another (Soler et al. 2008). In both the soil and above-ground compartments many organisms are associated with the plants, ranging from vertebrates and arthropods to micro-organisms. These organisms may have beneficial, neutral, or negative effects plant fitness. Plants have evolved a range of strategies to optimize associations with beneficial organisms and/or to prevent or reduce the negative effect of attack from their antagonists. Roots may harbour many antagonists and therefore it is important that plants do not only defend themselves in the shoots but in their roots as well (van Dam and Bezemer 2005; van Dam 2009). In a seminal paper, Ehrlich and Raven (1964) argued that plants and insects are involved in a sequential co-evolutionary arms race in which insect herbivores evolve strategies to deal with plant defences which are countered by new or stronger defences by the plant over evolutionary time. However, at the time their paper was written, the importance of this arms race was restricted to interactions between plants and insects in an above-ground framework. As we will explain here, this ‘arms race’ can also involve interactions between roots and root herbivores as well as indirect interactions involving root and shoot tissues and herbivores feeding on plant structures in opposite compartments.

In this paper we discuss studies investigating the processes and underlying mechanisms that underpin multi-trophic interactions with plants in the above- and belowground compartments (hereafter AG and BG). In particular, we broach a topic that has thus far received little attention in the empirical literature: factors generating and maintaining intraspecific variation in plant defence-related traits that are mediated through AG and BG trophic interactions. Plant defence traits often have a genetic basis. The effects of genetic variation in these plant traits on interactions with higher trophic level organisms have primarily been studied for AG plant parts (Crutsinger et al. 2006; Johnson 2008, Newton et al. 2009a, Utsumi et al. 2011). In the last decade, complexity and ecological realism in experimental designs has increased. This also includes genetic variation in the interacting AG and BG compartments and its effect on interactions with associated organisms (Rasmann et al. 2009; Vandegehuchte et al. 2011). With this paper we make a plea for a more holistic approach with respect to genetic variation and AG-BG interactions. First we give a brief overview of the literature on plant-mediated multi-trophic interactions in the AG and BG compartments and the hypotheses and underlying mechanisms that emerged from these studies. We then discuss the role of genetic variation in plant traits in shaping interactions with associated organisms. As an example, we give an overview of current knowledge on inter- and intraspecific AG-BG variation in plant defence traits and their consequences for interactions between insects and plant species in the large family Brassicaceae. We explore how different selection pressures at the species level may lead to the expression of variation in defence traits in roots and shoots using wild cabbage (*Brassica oleracea*) as our model species. We provide new data on root chemical defences, show how this compares with better-studied AG defences in this species, and highlight the importance of studying genetic variation in plant traits that play a role in AG and BG interactions with associated organisms in natural systems in order to explain the evolution and maintenance of variation among these traits.

Plant mediated above- and belowground interactions: patterns and hypotheses

Most studies involving plants and their defence traits in a bi- or multi-trophic framework have focused on the AG compartment (reviewed by Price et al. 1980; Karban and Baldwin 1997; Dicke 1999; Harvey 2005; Schoonhoven et al. 2005; Ode

2006; Hopkins et al. 2009). These studies have provided a wealth of data showing that direct and indirect plant defences can profoundly influence mechanisms governing species-level interactions and the structure of food webs up to (and perhaps even beyond) the fourth trophic level (Bukovinszky et al. 2008; Harvey et al. 2004, 2007). However, it is important to stress that plant-related traits, including defence, can also strongly influence biotic interactions BG (see reviews by Van der Putten et al. 2001; Van Dam et al. 2003; Bezemer and Van Dam 2005; Van Dam 2009; Van Dam and Heil 2011; Soler et al. 2012).

Given that plants may have to respond to variable stressors in both the AG and BG compartments, it is somewhat surprising that the importance and significance of interactions between these compartments has only emerged in the past 20 years or so. For example, studies by Gange and Brown (1989) and Masters and Brown (1992) showed that root herbivory by a root chewing insect was positively correlated with the pupal weight of a leaf mining insect. Masters (1995) found that leaf mining AG significantly decreased the performance of chafer larvae feeding BG, but at the same time root herbivory increased the pupal weight of the leaf miner. This positive influence of root feeding can also influence higher trophic levels. For instance, the abundance of a seed predator and two of its parasitoids were highest on thistle plants subjected to root herbivory (Masters et al. 2001).

It is now known that organisms in both compartments can indirectly influence each other through changes in the biomass, nutritional quality (primary metabolites) and chemical defence (secondary metabolites) of plants (Bezemer and Van Dam 2005; Van Ruijven et al. 2005). Recently, Kostenko et al. (2012) reported that in ragwort (*Jacobaea vulgaris*) herbivory by AG- and BG-feeding herbivores affects the soil fungal community, which in turn affects plant defence, biomass and multi-trophic interactions in ragwort plants in successive generations in different years. In many plant taxa secondary plant compounds are produced in the roots and then transported to AG plant structures (Karban and Baldwin 1997). Besides defence compounds, levels of nutritional metabolites, such as amino acids and carbohydrates, are often also affected by damage (Bezemer and Van Dam, 2005). The capacity of roots to absorb nutrients and the chemical composition of the soil are strongly affected by soil organisms. This affects the growth rate of plants, which is important in structuring plant communities and associated organisms (Van Dam and Heil 2011).

Differences in physical characteristics of the AG and BG compartments may have profound effects on the spatial and temporal processes and scales that shape interactions between plants and associated organisms across several trophic levels. It is generally far easier for plant antagonists and mutualists to disperse in the

AG than in the BG domain, since movement is clearly much more limited BG as a result of the simple physical difference between air and soil. For example, in the AG compartment, herbivores generally have easy access to plant parts, such as shoots and flowers, possibly resulting in intense short-term selection for defence-related (or in the case of pollinators, attraction-based) traits (Zangerl and Berenbaum 1993; Majetic et al. 2009; Parachnowitsch et al. 2012). As a result of these differences in the scale of AG-BG interactions, plants may have evolved variable responses to organisms in each compartment based on time differentials in the temporal sequences (and/or accumulative effects) of these antagonists. This temporal differential may lead many plants to evolve strong AG defences whereas they have evolved to 'tolerate' their BG antagonists until some critical threshold is reached whereby a plant population is forced to relocate to a new habitat (the 'above-ground selection, below-ground dispersal hypothesis') (Van der Putten et al. 2001, Bezemer et al. 2005). The release and perception of chemical cues, such as herbivore-induced plant volatiles (HIPVs), may also reflect differences between the AG and BG compartments. The rate and extent of transport of these cues are likely to be reduced BG. Furthermore concentrations of unspecialized and more specialized compounds may also differ between AG and BG plant tissues, and the dependency of chemical communication on water-soluble compounds is likely to be greater BG (Van der Putten et al. 2001).

Interactions between consumers in the AG and BG compartments have been very well studied in recent years (Anderson et al. 2011; Soler et al. 2012, 2013; Wurst 2010). Many of these studies have focused on elucidating mechanisms involving AG and BG organisms sharing the same plant (Erb et al. 2008). These interactions may vary in terms of complexity and may involve organisms from several trophic levels and functional groups or feeding guilds. Moreover, different genotypes of one plant species can differ in their response to BG or AG organisms (Wurst et al. 2008; Harvey et al. 2011), and BG and AG organisms themselves can respond differentially to plant genotypes (Crutsinger et al. 2006, Johnson 2008; Kabouw et al. 2011; Utsumi et al. 2011). The variable responses both in the plant and the herbivore make it difficult to predict the outcome of AG-BG plant-mediated interactions. For example, although plant genotype correlated positively with AG and BG invertebrate colonisation, correlations between the AG and BG invertebrate groups themselves were negative, suggesting that the two groups selected plant genotype differentially (Vandegehuchte et al. 2011).

The importance of higher trophic levels on herbivore-plant interactions was recognized first by Price et al (1980). Since AG-BG interactions may occur between organisms across several different feeding guilds and species, it is not surprising to find that the outcomes of these interactions may vary substantially from one association to another. Most AG-BG interaction experiments to date have focused on the effect of BG organisms on AG organisms, but there are also some studies that have looked at the effect of AG on BG (or both) (for a more in-depth discussion, see reviews by Soler et al. 2012, 2013). Plant-mediated AG-BG interactions may be decidedly non-linear, whereby small scale interactions between a plant and one type of organism can affect entire AG and BG food webs and communities associated with that plant (Bardgett and Wardle 2003; Wardle et al. 2004, 2005; De Deyn et al. 2007; Gerber et al. 2007; Heil 2011).

Several hypotheses have been proposed on the underlying mechanisms determining plant-mediated AG-BG interactions. The stress response hypothesis states that the removal of root biomass by root feeding organisms causes a similar response as drought stress (Masters et al. 1993). This results in an accumulation of soluble nitrogen and carbon in aboveground plant parts, thus increasing the nutritional quality of the plant for AG herbivores (Masters and Brown 1997). By contrast, the defence induction hypothesis posits that herbivores in the opposite compartments negatively influence each other through induction of toxic secondary plant compounds (Bezemer et al. 2003; Bezemer and Van Dam 2005). Because these compounds are often stored in the cells, phloem feeders will be less exposed to inducible toxic compounds, perhaps explaining why root feeders often negatively influence the performance of leaf chewers but not that of aphids. On the other hand, AG herbivores may negatively affect the growth and development of BG herbivores by reducing the availability of carbohydrates in the roots (Van der Putten et al. 2001). Using cotton plants, Bezemer et al. (2003) found no effect of previous feeding by a leaf chewing caterpillar *Spodoptera exigua* on the performance of root feeding *Agriotes lineatus* larvae. On the other hand, they found that root feeding by wireworms negatively affected the performance of *S. exigua*. Wurst and colleagues (2006) looked at the effect of two soil organisms on primary and secondary metabolites in cabbage and found that foliar concentrations of glucosinolates, secondary metabolites characteristic for Brassicaceous plants, was affected by these organisms. Earthworms decreased the concentration of glucoiberin in the plant shoots and interactions between earthworms and root-knot nematodes in turn affected concentrations of glucoraphanin. This may have an influence on AG herbivores, since glucoiberin can act as a feeding and oviposition stimulant, providing support for the defence induction hypothesis (Wurst et al. 2006). Another study also found a negative impact of root feeding on the oviposition and feeding behaviour of an aboveground herbivore (Anderson et al. 2011). More studies showed that root herbivory, through reduced plant quality, negatively affected the performance of AG herbivores, parasitoids and even hyperparasitoids (Van Dam et al. 2004a; Soler et al. 2005). AG herbivory by caterpillars of the large cabbage white butterfly, *Pieris brassicae*, negatively affected performance of a root feeding herbivore, the cabbage root fly, *Delia radicum* and its endoparasitoid, *Trybliographa rapae* (Soler et al. 2007a). Infestation of pepper plants with whiteflies elicited a BG defence response, resulting in reduced infection when exposed to AG and BG bacterial pathogens, whilst positively affecting the association of plant roots with beneficial micro-organisms (Yang et al. 2011).

Not only are plant-mediated AG-BG interactions modified by the feeding activity of arthropods, but also by the composition of the soil micro-fauna. A meta-analysis of studies investigating the effect of mycorrhizal fungi on the performance of insect herbivores showed that the mycorrhizal status of host plants is often ignored in studies, despite the fact that mycorrhizal fungi can induce morphological, physiological and biochemical changes and thus may influence plant quality for herbivores (Koricheva et al. 2009). In general, mycorrhizal fungi provide plants with nutrients and water and in return receive carbohydrates from the plant. The meta-analysis also revealed that phloem feeders benefited from mycorrhiza, whereas mesophyll feeders did not. The effect of dietary specialisation in combination with feeding mode was only significant for the chewing and not for sucking herbivores: specialist chewing herbivores performed better on plants colonized by mycorrhiza, whereas generalist chewing herbivores performed more poorly. In addition, mycorrhiza affected chewing herbivores negatively when these herbivores were feeding on the roots (Koricheva et al. 2009).

Bezemer et al. (2005) showed that the soil community composition can influence AG multi-trophic interactions by affecting plant nutritional quality. Inoculation with nematodes negatively affected aphid offspring production, and aphid population size was lowest in microcosms with both nematodes and microorganism. The reverse was found for the aphid parasitoids which performed best in microcosms with both nematodes and microorganisms (Bezemer et al. 2005). These examples clearly illustrate that there are many different outcomes that may be generated by AG-BG interactions.

Defining different types of genetic variation

Genetic variation can be studied at various levels of organization, from the expression of genes to individuals at the organismal level. Here, we have focused on genetic variation at the level of the individual plant. According to Whitham and colleagues (2003), in order to better understand interactions between species and communities, genetic variation should be divided in three classes: (1) genetic variation within single populations of the same species, (2) genetic variation between different populations of the same species, and (3) genetic variation among different species.

Genetic variation is known to be expressed in many different plant traits, including morphology, phenology, primary and secondary chemistry. The expression of specific secondary metabolites is often taxonomically constrained (Schoonhoven et al., 2005). For example, different plant families are often characterised by their own classes of secondary metabolites, e.g. alkaloids in the Solaneceae, benzoxazinoids in the Poaceae and glucosinolates in the Brassicaceae.

Intra-specific genetic variation in plant AG defence traits and its effects on the behaviour and/or development of herbivores and their natural enemies in both lab and field studies has been well studied. In particular, much is known about this field of research in cultivated and wild plant species in the Brassicales, which includes cabbages, mustards and related crops and their wild relatives. This includes *Arabidopsis thaliana* (Bidart-Bouzat and Kliebenstein 2008; Wentzell and Kliebenstein 2008), *Brassica nigra* (Lankau and Strauss 2007, 2008), *B. rapa* (Pilson 1996, 2000), *Raphanus raphanistrum* (Agrawal et al. 2002), and both wild (Harvey et al. 2007, 2011; Gols et al. 2008a,b; Newton et al. 2009a,b) and cultivated (Poelman et al. 2008; Kos et al. 2011) *B. oleracea*. These studies and others with different plant taxa have generated a wealth of mechanistic data showing the reciprocal effects of genetic variation in AG plant defence traits on consumers up the food chain, as well as both biotic and abiotic factors that may be driving this variation (Crutsinger et al. 2006; Johnson 2008; Newton et al. 2009a; Utsumi et al. 2011).

Genetic variation is usually based on trade-offs involving the costs and benefits of retaining certain traits when metabolic resources are limiting (Stearn, 1992). For example, trade-offs may occur in resource allocation between defence traits and growth (e.g. competitive ability). This has been reported in a number of invasive plants when released from their co-evolved native enemies (e.g. pathogens and herbivores) in their new ranges. In this situation, plants quickly reallocate metabolic resources from defence to growth, meaning that they are able to out-compete native vegetation (Zangerl and Berenbaum 2005; Wolfe et al. 2004). This rapid switch from defence to growth supports the predictions of the 'enemy-release' and 'evolution of increased competitive ability' hypotheses (Maron and Vila 2001; Keane and Crawley 2002; Joshi and Vrieling 2005; Colautti et al. 2004). Within the group of plant defence traits, there are also numerous trade-offs. The different defence traits of plants may conflict because of their energy demand (Van der Putten et al. 2001).

Storing valuable resources in the roots can make a plant less attractive for AG herbivores, but it will make the roots more attractive to BG herbivores. Re-allocating resources from roots to shoots and leaves may increase resistance to AG herbivory, but also means limited capacity of the roots to establish/maintain mutualisms with

BG microorganisms (Heil 2011). Among populations, different plant traits can be selected for, depending on the local conditions. The resulting local adaptation means that individual plants have a higher fitness at their home site compared to other sites inhabited by the same species (Kalske et al. 2012). Trade-offs in local adaptation can be caused by limited resources, allocation costs or ecological or genetic constraints (Kalske et al. 2012). Thus far, trade-offs in various defence related traits in plants in response to combined AG and BG biotic interactions has received little attention, and therefore is a fertile area for future research (but see Vandegehuchte et al. 2011). It is important to keep in mind that various plant traits are not necessarily costly to maintain or, conversely, only have weak (or no) effects on plant fitness, in which case it is unlikely that adaptation will occur.

Genetic variation in defence and other traits in plants

Evolution can only take place when natural selection acts on genetic variation in heritable traits that affect fitness (Whitham et al. 2003; Hughes et al. 2008). Without heritable phenotypic variation, there is no adaptive evolution possible. It is therefore important to determine what factors generate and maintain genetic variation within and between different populations (Siepielski and Benkman 2009). Important sources for genetic variation in plants are introgression, mutation and recombination at the gene level (Siepielski and Benkman 2009), and also gene flow and genetic drift at the population level. The fact that there is heritable trait variation does not automatically mean that different levels of genetic diversity have predictable ecological consequences, because other factors (e.g. the environment) also play an important role (Hughes et al. 2008).

Genetic variation in plant defence traits is driven by a number of biotic and abiotic factors that may well be synergized (see discussion below with wild cabbage to get a better perspective). Much attention has been paid to trophic interactions between plants and their antagonists such as pathogens and herbivores, often in a co-evolutionary framework. Indeed, co-evolutionary theory underpins our understanding of intimate consumer-resource interactions in nature (Pimentel 1961; Ehrlich and Raven 1964; Rosenzweig 1973; Abrams 1986; Marrow & Cannings 1993; Bonte et al. 2010; de la Pena et al. 2011). Many of the classical studies on co-evolutionary arms races and adaptive radiation have explored interactions between insect herbivores and their food plants (Ehrlich and Raven 1964; Benson et al. 1975; Berenbaum & Zangerl 1992; Hamrick and Godt 1996; Pilson 1996, 2000; Janz and Nylin 1998; Lankau 2007; Lankau and Strauss 2007; Cogni and Futuyma 2009; Becerra et al. 2009; Carmona et al. 2011; Bode and Kessler 2012; Holeski et al. 2012; Bernhardsson et al. 2013). More recently it has been argued that selection for certain traits occurring in pair-wise fashion are often generated at local or small landscape scales, and the term evolutionary 'hotspots' has been invoked to describe this phenomenon (Thompson 2005a). In this situation localized populations of closely interacting species interact intensively in small, often isolated patches and thus evolve unique traits that reflect adaptations to one another: one (the consumer) to exploit and one (the resource) to resist. Given that selection intensity can vary depending upon local conditions, evolutionary hotspots may be distributed over space and time as 'geographic mosaics' (Thompson 2005a). Thus far, however, discussion of selection pressures generated in hotspots has focused on the AG domain, with little effort to determine if and to what extent selection can occur from combined AG-BG interactions. To study this it is necessary to measure genetic variation in the expression of AG-BG plants traits and to determine if they are correlated (see e.g. Kaplan et al. 2008). Moreover, field studies are needed to identify and measure qualitative and quantitative differences in AG and BG communities associated with a plant at different spatial and temporal scales. In addition, selection on certain plants may be characterized by diffuse selection (Vandengehuchte et al. 2011; Strauss and Irwin 2004). Alternatively, herbivores may respond to variation in defence traits without exerting any selection pressures themselves.

As described above a plethora of studies have examined the biotic factors driving selection for AG defence traits in plants, and in particular allelochemistry (Coley et al. 1985; Schoonhoven et al. 2005). For example, Zangerl and Berenbaum explored whether herbivores can select for rapid increases in secondary metabolites (xanthotoxins) in plants, using wild parsnip and its main herbivore, the parsnip webworm, *Depressaria pastinacella* as a model system. This plant species harbours few herbivores in nature, aside from *D. pastinacella*, whose larvae attack seeds and thus may greatly affect plant fitness (Zangerl and Berenbaum 1993). Wild parsnip has been introduced into various parts of the world where it has become an invasive pest in some areas (Berenbaum et al. 1986). In some regions where it has been introduced, webworms have also been released as a means of biological control, although in many habitats where the plant is established these herbivores are still absent. The main secondary metabolites in *P. sativa* are furanocoumarins, toxic compounds found primarily in species of the Apiaceae and Rutacea. Zangerl et al. (2008) showed that in areas where webworms are absent, parsnips rapidly responded by reducing investment in chemical defences, suggesting that they are costly to maintain (see also Berenbaum & Zangerl 2006). However, when webworms were introduced into regions where parsnips had been established for some years, the plants rapidly responded by reallocating metabolic resources to the production of furanocoumarins, showing that rapid evolutionary responses to chemical defences are possible (Berenbaum and Zangerl 1998).

The role of higher trophic levels, such as predators and parasitoids, in driving selection of plant-related traits has received less attention, although it has been amply demonstrated that natural enemies can significantly reduce herbivore abundance in agricultural landscapes (Luck et al. 1988; DeBach and Rosen 1991). Cropping systems are often characterized by monocultures of plants whose direct chemical defences have been greatly reduced as a result of artificial selection via domestication (Gols and Harvey 2009). Natural systems are generally much more complex than agro-ecosystems chemically and structurally. How important trophic cascades involving insects are, has been the subject of debate (Hairston et al. 1960; Huntly 1991; Schmitz et al. 2000). Evidence is coming to light that investment in costly plant secondary metabolites can be significantly influenced by the presence or absence of parasitoids, and that these effects generate phenotypic mosaics at the landscape-scale. Once again, the best studied system in which this area has been explored is the *P. sativa* - *D. pastinacella* association. Work by Berenbaum, Ode and colleagues has found that one parasitoid species, the encyrtid wasp *Copidosoma sosares*, devastates *D. pastinacella* populations where both species along with the food plant are native in western and central Europe (Ode et al. 2004; Ode 2006; Lampert et al. 2008; Berenbaum and Zangerl 2006). Where all three species are common in the native range, *P. sativa* plants are apparently less toxic than in areas of the invasive range where only the plant and herbivore have been established (Berenbaum and Zangerl 2006). However, when plants and herbivores in the invasive range have been reunited with *C. sosares*, the plants quickly lower investment into the production of furanocoumarins, presumably because the parasitoids are again greatly reducing levels of herbivory (Berenbaum and Zangerl 2006). Future studies comparing defence traits in populations of native and invasive plants in a multi-trophic framework incorporating natural enemies offer much promise in better understanding rapid shifts in traits, such as from defence to growth. More importantly, future studies need to explore this combining AG and BG compartments, given what we already know about the importance of this linkage.

Where herbivores might select for high chemical defence levels, competition between plants might select for other plant traits although some of these may also involve phytotoxins (e.g. in the case of allelopathy). This is a complex matter, since plants compete not only with other plant species but also with conspecifics. For example, sinigrin produced by *B. nigra* is allelopathic and retards the germination and growth of wild oat and wild barley (Turk and Tawaha 2003; Tawaha and Turk 2003). Lankau and Strauss (2008) stated that, due to the costs of trait maintenance, a trait that improves interspecific competition will at the same time reduce intraspecific competition. Sinigrin is costly to produce and functions not only in competition with other plants but also as a defence compound against herbivores and pathogens.

In *B. nigra* there is a negative genetic trade-off between inter- and intraspecific competitive ability (Lankau and Strauss 2008). When competing with different plant species, genotypes that produced high concentrations of sinigrin were strong competitors. However, in competition with conspecifics, these genotypes did poorly because there were no benefits to be gained from producing high concentrations of sinigrin (Lankau and Strauss 2008, Lankau et al. 2011). While interspecific competition is primarily influenced by the allelopathic and anti-mycorrhizal effects of sinigrin, intraspecific competition is based on resource capture (Lankau and Strauss 2007). Lankau and Strauss (2008) also found that the assembly of plant and herbivore species present in a community influence selection pressures acting on the production of sinigrin. Importantly, the identity of the competing plants affected selection for sinigrin production in the presence of herbivores more than the number of neighbouring plants. Thus, the associated plant and herbivore community acts as an important and variable selection pressure on sinigrin in black mustard. Johnson (2008) studied the effect of plant genotype on intra-specific competition between evening primrose (*Oenothera biennis*) plants. This plant species exhibits heritable variation in above- and belowground growth and different genotypes responded differently to competition. Although evening primrose affected other plants through competition in the greenhouse, it was found that soil fertility had a much stronger effect and that in the field, there was no genotypic effect on neighbouring plants. Johnson concluded that in this case environmental variation was a stronger determinant of competition than plant genotype (Johnson 2008).

Although this area has been little studied, we argue that interactions between plants and AG and BG organisms may influence the evolution of traits such as defence against herbivores, attraction for pollinators, as well as competition between plants for access to water, nutrients and light. The way these interactions are played out in natural communities can affect plant fitness. For example, Poveda and colleagues (2003) looked at the separate and combined effects of root and leaf herbivory on plant fitness in charlock mustard, *Sinapis arvensis*. Root herbivory marginally increased the flowering period and number of fruits produced when compared with combined root and leaf herbivory. It was also correlated with a higher *per capita* number of flower visits by pollinators (Poveda et al. 2003). These finding are in contrast with other studies where a negative effect of root herbivory on plant growth (Gange and Brown 1989) or reproduction (Masters et al. 2001) was found, although in the Poveda et al. (2003) study this may have been caused by the low number of root herbivores per plant.

Barber et al. (2011) examined the effect of AG and BG herbivory on the performance of cucumber plants and found that, although root herbivory positively affected flower visitation by honey bees, root herbivory had a stronger negative effect on plant reproduction than leaf herbivory. Moreover, plant growth was reduced by both leaf and root feeding, whereas flower production was negatively affected by root herbivory resulting in less female flowers. Maron (1998) studied the effects of AG and BG insect herbivores on bush lupine and found that they had the potential to impose strong selection on the plant in several ways: via the suppression of AG herbivores that increased seed production and via the suppression of BG herbivores that also increased seed production but which additionally decreased plant mortality. The results of these experiments shed some light on the complexity of AG-BG interactions and plant fitness and suggest that in terms of selection regimes the effects may not only be association-specific but also vary in different populations at the landscape scale.

Not only do herbivores exert selection pressures on plants, but it also works the other way around. Because plants and specialist herbivores are often involved in a co-evolutionary arms race, anti-herbivore defences of plants may select for herbivore genotypes that are best able to deal with those defences and vice versa (Kant et al. 2008). For example, spider mites dealing with jasmonic acid in tomato plants developed three different genotypes that differentiated in their induction of and resistance to jasmonic acid-induced defences (Kant et al. 2008). Some populations of a perennial herb had associated herbivores that were locally adapted to their genotype, but in other populations the plants were adapted to the herbivores (Kalske et al. 2012). It remains to be determined at larger spatial (= geographical) scales if differences in combined AG- BG selection pressures can drive genetic variation in plant responses at the species and population level, as reflected in the measurement of different traits such as growth and defence. Moreover, how AG and BG plant responses in combination can drive reciprocal selection in herbivores and perhaps even their natural enemies is largely unknown (but see Vandegehuchte et al. 2011; Bonte et al. 2010). Although this infers the expression of some immensely complex processes that span several to many links, there is no reason that such effects do not occur in habitats where there are strong frequency-dependent AG and BG interactions.

Defence chemistry in wild cabbage, *Brassica oleracea*, and related species

Plant species in the Brassicaceae are well studied with respect to (genetic) variation in secondary plant chemistry (Halkier and Gershenzon 2006; Agerbirk and Olsen 2012) and their interactions with AG and BG insect herbivores, but also with species in the third and even the fourth trophic level (Harvey et al. 2003; Soler et al. 2005;

Soler et al. 2007b; Gols and Harvey 2009; Hopkins et al. 2009). Secondary metabolites characteristic for plants in this family are the glucosinolates (hereafter GS). They are sulphur- and nitrogen-containing plant secondary metabolites that can be divided in three different classes based on their amino acid origin: aliphatic, indolyl and aromatic GS (Halkier and Gershenzon 2006). When plant tissues are disrupted by for example insect feeding, myrosinase enzymes come into contact with the intact GS, and hydrolyse them into various hydrolysis products. Especially these GS breakdown products play a role in defences against various attackers such as generalist insect herbivores and pathogens (Mithen 2001). However, as specialist insect herbivores have evolved efficient mechanisms to excrete, detoxify or sequester GS (Bridges et al. 2002; Ratzka et al. 2002; Wittstock et al. 2004; Müller 2009a), they may use GS and their breakdown products as stimuli to recognise host plants for oviposition and feeding (Renwick 2002; Renwick et al. 2006; Bidart-Bouzat and Kliebenstein 2008). Plant quality for specialist herbivores is determined by more general plant characteristics such as levels of primary metabolites and mechanical traits (Travers-Martin and Muller 2008).

Various species of *Brassicas* differ in their GS profiles, both in AG and BG tissues (Figure 1A and 2A). For example, the relative GS concentrations in AG and BG tissues differ dramatically, with root concentrations being much higher than shoot concentrations in *Bunias orientalis,* these being lower in *B. nigra* and similar in *S. arvensis* (Figure 1B). Usually, levels of GS are lower in BG than in AG tissues van Dam 2009. Across species, variation in defence chemistry has been demonstrated to affect the performance of associated insects (Francis et al. 2001; Müller et al. 2002; Renwick 2002; Harvey et al. 2003; Gols et al. 2008a; Harvey et al. 2010). These dramatic differences in plant secondary chemistry at the species level may have implications for the interactions with other organisms in nature.

Although GS have been shown to play an important role in protecting plants against generalist insect herbivores (Blau et al. 1978; Gols et al. 2008b), other studies have shown that this is not always the case. For example sinalbin, the dominant GS in both AB and BG tissues in *B. orientalis* (Figure 1B), appears not to be effective against feeding by the generalist herbivore *Mamestra brassicae* (Harvey and Gols 2011a). Moreover, the high GS concentrations in BG tissues in this invasive species may play a role in its competitive abilities with other plant species or soil organisms that negatively affect growth and development of this plant and may explain its invasion success, but this needs to be tested empirically (Müller 2009b). Remarkably, *B. orientalis,* which is readily accepted for oviposition, is a poor food plant for all studied specialist herbivores (Harvey et al. 2010). This result suggests that other chemicals in *B. orientalis* render these plants unsuitable for development of specialist herbivores.

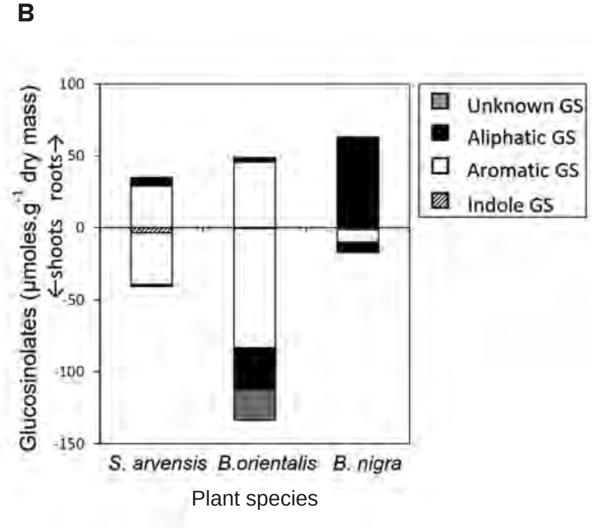

Figure 1A *Sinapis arvensis* (a), *Bunias orientalis* (b) *and Brassica nigra* (c)

Figure 1B Mean (n=10) shoot and root (negative values) glucosinolate levels in undamaged greenhouse-grown *Sinapis arvensis, Bunias orientalis, Brassica nigra* originating from natural growing populations in the Netherlands (Glucosinolates were classified according to their amino acid origin into indole, aromatic and aliphatic GS. The dominant GS in *S. arvensis* and *B. orientalis* was the aromatic GS sinalbin, whereas the dominant GS in *B. nigra* was the aliphatic GS sinigrin. The root tissues of *B. orientalis* also contained relatively high levels of an unknown GS (see van Dam et al. 2004 for analysis methods).

Brassica oleracea is native to the coastlines, especially calcareous cliffs, of Western Europe and is considered the progenitor of cultivated cabbage (Mitchell and Richards 1979). In the UK, the largest populations are on the south-west coast in the counties Cornwall, Devon and Dorset (Wichmann et al. 2008). The distribution of the wild cabbage populations along the Dorset coast has been very constant over the past 70 years (Wichmann et al. 2008). These populations have also been the subject in a number of studies investigating the variation in GS metabolites, as well as the factors that maintain this variation considering that these populations grown often less than 15 km apart. The differences in GS profiles are most likely caused by divergent abiotic and biotic selection in the different habitats (Mithen et al. 1995; Moyes et al. 2000; Moyes and Raybould 2001; Newton et al. 2009b; Newton et al. 2010), although random processes such as founder effects and genetic drift may have played a role here as well.

Variation in the expression of GS is not only expressed across, but also within species. Populations of wild cabbage (Figure 2A) differ considerably in their GS profiles (Figure 2B) with concomitant consequences for the performance of insects in the second and third trophic level (Gols et al. 2008b; Harvey et al. 2011). Moreover, GS concentrations change in response to herbivory or simulated herbivory (see also van Dam et al. 2004) and population related differences in induction, although not found here (Figure 2A), have been reported for the wild cabbage populations when induced by a different herbivore *Pieris brassicae* (Harvey et al. 2011). The variation in GS concentration appears to be more pronounced in AG than in BG tissues (Figure 2B). For example, indole GS dominate the profile in leaf tissues of the Kimmeridge population, whereas leaves sampled from Winspit plants contain high alkenyl GS concentrations. In the roots of the three populations all GS classes are represented and levels are relative little affected by induction compared to induction of foliar tissues.

Mithen et al. (1995) suggested that herbivores could act as an important selective force driving GS variation in the wild cabbage populations. However, Moyes et al. (2000) argued that for herbivores to act as a selection force, the herbivores need to select individual plants based on their GS profiles. They showed that there was a potential for host plant selection based on differences in the GS profiles of neighbouring plants in the population on a small scale, but found no correlation between herbivore preference and GS profile except for one specialist herbivore species. They made the point that although laboratory experiments showed that GS influence the performance of herbivores, there was little evidence that this was also the case in nature (Moyes et al. 2000). In contrast, Newton et al. (2009a) reported significant differences in the response of herbivores to aliphatic GS, both within and between plant populations in the field. Based on their findings, they concluded that variation in GS can structure the associated herbivore community (i.e. herbivore mediated differential selection, Newton et al. 2009a). However, to demonstrate herbivore-mediated differential selection conclusively, further evidence is required showing that variable attacks by herbivores in the field have consequences for plant fitness (Newton et al. 2009a).

A

Figure 2A *Brassica oleracea*

B

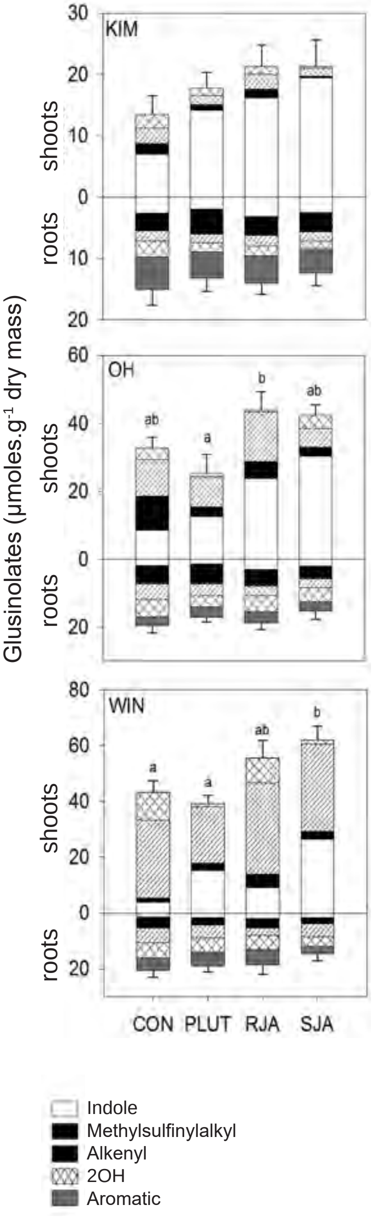

Figure 2B Shoot and root glucosinolate levels (mean + SE of mean total, n=4 or 5) of *Brassica oleracea* plants originating in Dorset, England from three wild populations located at sites called Kimmeridge (KIM), Old Harry (OH) and Winspit (WIN), respectively. Glucosinolates were classified according to their amino acid origin into indole, aromatic and aliphatic GS. The latter group was further divided into methylsulfinyl, alkenyl, and hydroxyl (=OH) GS. The plants were either untreated controls (CON), induced with 9 second instar *Plutella xylostella* (PLUT) larvae divided over three leaves, induced with 500 µg jasmonic acid either applied to the roots (RJA) or to the shoots (SJA). Jasmonic acid was used to simulate herbivory by chewing herbivores (van Dam et al. 2004). Roots and shoot tissues were harvested for GS analysis 7 days after the induction treatments. Different letter over the bars indicate significant differences ($P < 0.05$) in total glucosinolate level between the bars within each panel (Tukey HSD multiple comparisons among means). Please note the difference in scaling of the X-axes. Both population and induction treatment had a significant effect on total GS levels in wild *B. oleracea* (MANOVA, treatment $F_{6,82} = 5.77$, $P < 0.001$; population $F_{4,82} = 18.7$, $P < 0.001$). All classes of GS, as well as total GS concentrations, differed with population origin in both the roots and the shoots ($P<0.05$ for all analyses). In the shoots, indole GS ($F_{3,42} = 23.9$, $P < 0.001$) increased in response to the three induction treatments. Aromatic GS were also affected by induction treatment ($F_{3,42} = 3.34$, $P = 0.03$). Only WIN shoots contained small amounts of aromatic GS and these decreased with shoot induction, *P. xylostella* feeding and JA treatment, but increased with root JA application. In the roots, only indole GS responded significantly to induction treatment ($F_{3,42} = 7.57$, $P < 0.001$); JA applied to the roots increased indole GS levels in these tissues.

The populations along the Atlantic coast of England are known to be exposed to different abiotic conditions, despite their relative close proximity to each other. While some populations are located on high cliffs and thus are fully exposed to the prevailing wind, others are located in sheltered valleys. This may affect the colonisation of plants by herbivores and their natural enemies, with populations on the cliffs experiencing low and population in the valleys experiencing high insect pressures. Soil characteristics such as clay and water content, soil texture, and nutrient levels have been reported to differ among the wild cabbage sites (Mithen et al., 1995; Wichmann et al. 2008). Little is known about the biotic selection pressures BG that may explain the relative lower variation in root GS chemistry of the wild cabbage populations. We currently investigate variation in associated soil communities at several of the wild cabbage sites in Dorset in order to reveal the degree of biotic BG variation.

The 'Geographic Mosaic of Co-Evolution Theory' predicts that the intensity of selection pressure exerted by herbivores on plants may vary geographically (Thompson 2005). Local differences in selection pressure may thus result in population-related variation in the expression of certain traits. The ultimate question with respect to the wild cabbage populations is what processes maintain this high level of variation in secondary chemistry and potentially other traits and whether this variation is the consequence of strong selection pressures exerted locally. In addition, selection pressures may differ with respect to the AG and BG compartment. In other words, spatial heterogeneity in defence traits may be expressed differentially in AG and BG tissues as a result of differences in selection pressures in the two compartments. Moreover, the third trophic level as a selection force BG should be included as well (Price et al. 1980). In agricultural fields, cabbage root flies (*Delia radicum*) cause considerable damage to cabbage crops and they are also known to be attacked by various parasitoids species. Foraging behaviour of parasitoids of *Delia radicum* has been reported to be affected by caterpillar feeding AG (Pierre et al. 2011). These results suggest the importance of a holistic approach of AG-BG multi-trophic interactions.

Conclusion and future directions

The study of AG-BG multi-trophic interactions is now a major area of research in ecology. Over the past two decades a significant amount of empirical data has demonstrated the importance of AG-BG interactions in terms of mechanisms relating to the behaviour and development of insects and other invertebrates, as well as effects on community structure and food webs. As the field continues to blossom, it is hoped that links between AG and BG compartments can be used to explain important applications in ecology, such as the production and delivery of important provisioning ecological services, e.g. the maintenance of soil fertility, nutrient cycling, pollination and even regional climate control. There is little doubt that a more intensive multi-disciplinary approach to the study of AG and BG ecology will yield many insights into the functioning of ecological systems and their role in sustaining human civilization.

At present, however, there are still some significant gaps in our knowledge of important mechanisms and processes, such as in the spatio-temporal variation in AG-BG interactions and in how they may drive selection for different plant-related traits such as defence and competitive ability. Furthermore, we are only beginning to scratch the surface in our understanding and appreciation of the role played by natural enemies in generating variation in various plant traits. Given the potential importance of trade-offs between tolerance (growth) and defence in plants, the influence of natural enemies such as parasitoids in driving selection may be vastly underappreciated. If we incorporate natural enemies of plant antagonists in the soil, and then link these with 3 or even 4 trophic level interactions AG, there is a potential wealth of outcomes that remains to be explored. Moreover, given what we now know about evolutionary hotspots where selection is played out intensively, it would be interesting to search for these hotspots in a plant species within and between habitats, and to try and match phenotypes with strong AG, BG and combined (AG and BG) selection regimes. Furthermore, given that plants can also potentially drive genetic variation in their associated consumers over several trophic levels and via multiple linkages, it would be interesting to explore how this may be played out combining AG and BG interactions.

We suggest several areas for future investigations:

(1) Studies working with different genotypes of wild plants and determining how these affect the behaviour and performance of AG and BG insect herbivores and their natural enemies associated with them both independently and in combination;
(2) Analysing various plant traits in roots and shoots in the same plant species both within and between populations along a geographical transect where abiotic and biotic selection pressures may vary. Furthermore, working to determine how differences in these traits are correlated with selection pressures from antagonists in the roots and shoots;
(3) Searching for geographical 'hot-spots' in which selection for AG and BG responses are rigidly enforced and the interactions with the various consumers are identified;

(4) Comparing AG and BG interactions in geographically widespread plants both in the native and invasive ranges, and determining how release from their co-evolved natural enemies AG, BG (or both) may have led to a relaxation in selection for defence-related traits. Studies with invasive plants have generally ignored links between AG and BG trophic interactions, which may be a major omission in understanding why a small percentage of exotics become invasive pests.

In summary, we argue that the field of AG-BG multi-trophic interactions needs to explore a wider range of biotic and abiotic selection pressures in explaining genetic variation in plant-related traits (and also reciprocally in their consumers up the food chain). In doing so it will be possible to develop a more thorough appreciation of the questions underpinning the immense variation in traits expressed in plants at various spatial scales.

Chapter 3

Development of a generalist predator, *Podisus maculiventris*, on glucosinolate sequestering and non-sequestering prey

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Published in: *Naturwissenschaften* 101: 707-714 (2014)

Abstract

Insect herbivores exhibit various strategies to counter the toxic effects of plant chemical defences. These strategies include the detoxification, excretion and sequestration of plant secondary metabolites. The latter strategy is often considered to provide an additional benefit in that it provides herbivores with protection against natural enemies such as predators. Profiles of sequestered chemicals are influenced by the food plants from which these chemicals are derived. We compared the effects of sequestration and non-sequestration of plant secondary metabolites in two specialist herbivores on the development of a generalist predator, *Podisus maculiventris*. Profiles of glucosinolates, secondary metabolites characteristic for the Brassicaceae, are known to differ considerably both inter- and intra-specifically. Throughout their immature (= nymphal) development the predator was fed on larval stages of either sequestering (turnip sawfly, *Athalia rosae*) or non-sequestering (small cabbage white butterfly, *Pieris rapae*) prey that in turn had been feeding on plants originating from three wild cabbage (*Brassica oleracea)* populations that have previously been shown to differ in their glucosinolate profiles. We compared survival, development time and adult body mass as parameters for bug performance. Our results show that sequestration of glucosinolates by *A. rosae* only marginally affected development of *P. maculiventris*. The effects of plant population on predator performance were variable. We suggest that sequestration of glucosinolates by *A. rosae* functions not only as a defensive mechanism against some predators, but may also be an alternative way of harmlessly dealing with plant allelochemicals.

Introduction

Plant-insect interactions have long been considered as model systems for studying the concept of co-evolutionary 'arms races'. In this scenario, plants evolve mechanisms to repel attacking herbivores or to negatively affect their performance, whereas the herbivores evolve counter mechanisms that allow them to effectively circumvent these defences (Ehrlich and Raven 1964). There is widespread consensus that the high degree of dietary specialization exhibited by insect herbivores is the direct consequence of this co-evolutionary arms race (Jaenike 1990; Mitter et al. 1991; Loxdale et al. 2011). In this scenario, generalists lose out because they are unable to deal with defences produced by different plant taxa that are phylogenetically conserved.

Plants exhibit two main defensive strategies in protecting themselves against herbivore attack: morphological (e.g. the production of trichomes or waxy leaf surfaces that impede herbivore movement and colonization) and chemical defences (e.g. the production of toxic or repellent secondary compounds). The effects of plant secondary metabolites on herbivore performance are often manifested through negative effects on their development and fitness, including increased mortality (Macel et al. 2005; van Dam et al. 2005), extended development time (van Dam et al. 2005; Reudler et al. 2011), reduced growth rate (Vrieling et al. 1991; Biere et al. 2004; Coley et al. 2006) or lower adult body mass (Harvey et al. 2005). Such negative effects can trickle up to the third trophic level or even higher (Harvey et al. 2003; Ode 2006). Plant traits, including chemical defences, often vary considerably both within and between different populations, which may have consequences for arthropod communities associated with these plants (Zangerl and Berenbaum 2005; Agrawal et al. 2012; van Geem et al. 2013).

In response to plant chemical defences, insect herbivores have evolved a variety of counter adaptations to reduce or negate their harmful effects. These adaptations include detoxification, excretion and sequestration of plant allelochemicals (Nishida 2002; Renwick 2002; Müller 2009; Winde and Wittstock 2011). Each of these areas has been well studied. For example, parsnip webworms are able to detoxify allelochemicals produced by the wild parsnip (Zangerl and Berenbaum 2003). On the other hand, green peach aphids (*Myzus persicae*) actively excrete allelochemicals, which they ingest through phloem sap, in their honeydew (Merritt 1996). Many insects are also known to harmlessly assimilate plant toxins into their own body tissues where they provide an additional benefit of being putative defences against natural enemies, and in particular generalist predators (Fordyce 2001; Nishida 2002; Müller and Arand 2007). Indeed, existing theory suggests that this is the main function of sequestration (Trigo 2000; Opitz and Müller 2009).

There are three ways in which secondary metabolites can be sequestered: absorption through the gut membrane, direct transportation into the haemolymph, or deposition into certain body parts (Nishida 2002). Because of their potential repellency or toxicity, many sequestering insect herbivores advertise their unpalatability through the production of bright body coloration (typically red, orange or yellow and black) that contrasts with the generally green background of their food plants (or habitats) in nature. A classic example of warning coloration and unpalatability is demonstrated in both immature and adult stages of the Monarch butterfly, whose specialist-feeding larvae sequester highly toxic cardiac glycosides from milkweeds (Malcolm and Brower 1989; Nishida 2002, Holzinger et al. 1992). Many other examples of sequestration have been shown in both generalist and specialist-feeding herbivores (Fordyce 2001; Hartmann et al. 2004; Singer and Bernays 2009; Lampert et al. 2011).

In this study, we examine the performance of a naïve insect predator, the spined soldier bug, *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae) developing on two herbivore species, one which sequesters plant allelochemicals and one which does not. The turnip sawfly, *Athalia rosae* (L.) (Hymenoptera: Tentredinidae) is a specialist herbivore of Brassicaceous plants (Müller et al. 2002). Larvae of *A. rosae* feed in loose assemblages on the food plants and sequester glucosinolates (hereafter GS), secondary metabolites that are characteristic of plants in the family Brassicaceae (Gols et al. 2008b; Hopkins et al. 2009). The larvae are grey to black in colour and are thus poorly camouflaged when feeding. The larvae are also known for their 'easy bleeding' whereby they voluntarily rupture part of their integument and produce a droplet of haemolymph when they are attacked (Boevé and Schaffner 2003). The haemolymph acts as a feeding deterrent for insect predators and has also been shown to be toxic to ants (Müller et al. 2002; Boevé and Müller 2005). In contrast with larvae of *A. rosae*, caterpillars of the small cabbage white butterfly, *Pieris rapae* (L.) (Lepidoptera: Pieridae) do not sequester GS (Müller et al. 2003). Instead, the caterpillars produce a 'nitrile-specifier protein' that detoxifies the GS by forming harmless nitriles which are excreted with the faeces (Wittstock et al. 2004). All instars of *P. rapae* larvae are light green in colour and thus are well camouflaged when feeding on the host plant.

Here, the herbivores were reared on three different populations of wild cabbage plants that are found along a small linear transect of the Dorset coastline in the U.K. The type and quantity of GS produced by plants from these three populations vary significantly (Moyes et al. 2000; Gols et al. 2008b; van Geem et al. 2013). Previous studies with wild cabbages have shown that the performance of generalist and specialist herbivores, including *P. rapae* and *A. rosae*, and parasitoids of *P. rapae*, differ significantly when reared on the three wild cabbage populations (Harvey et al. 2007; Gols et al. 2008a,b; Harvey and Gols 2011; Harvey et al. 2011; Abdalsamee and Müller 2012).

GS are well-studied in relation to (multi-trophic) plant-insect interactions (Harvey et al. 2003; Gols and Harvey 2009; Soler et al. 2012). They are known to act as feeding deterrents (Giamoustaris and Mithen 1995) or to have directly toxic effects on a range of plant enemies including pathogens, nematodes and insect herbivores (Hopkins et al. 2009). In Brassicaceous plants the chemical mechanism is based on the GS-myrosinase (hereafter MYS) system (Winde and Wittstock 2011). In intact plant tissues, GS and the enzyme MYS are stored separately. When GS come into contact with the MYS (e.g. through feeding damage of herbivores and resulting cell disruption), they hydrolyse and produce toxic breakdown products such as isothiocyanates (Winde and Wittstock 2011).

The main aim of the current study is to compare the development, in terms of survival, development time from L1 nymph to adult and adult body mass of *P. maculiventris* when reared exclusively on the GS-sequestering (*A. rosae*) or non-sequestering (*P. rapae*) prey. Based on previous work and on existing theory with respect to the defensive function of sequestration, we hypothesize that the performance of the predatory bug would be better on *P. rapae* then on *A. rosae*.

Material and Methods

Plants and insects

Brassica oleracea. Wild cabbage seeds were collected from three different populations: Kimmeridge (KIM), Winspit (WIN) and Durdle Door (DD), which grow naturally along the south coast of the UK, near Swanage, Dorset, U.K. (KIM: 50^{0}35′N, 2^{0}03′W; WIN: 50^{0}34′N, 2^{0}02′W; DD: 50^{0}62′N, 2^{0}27′W). Experimental plants were grown from these seeds in a glasshouse in the Netherlands. From each population 75 seeds were germinated in small plastic boxes containing germination soil (‘Lentse Stekgrond’, Lent, The Netherlands). After one week seedlings were individually transplanted to 2-L pots containing a soil mixture of 11% clay, 69% peat and 20% pumice. Four weeks later, a second batch of seeds was germinated to ensure that enough plant material was available for the insect herbivores for the total duration of the experiment. The plants were grown in a glasshouse at 21±1 ^{0}C during the photoperiod of 16h and 16±1 ^{0}C during the scotophase of 8h and a relative humidity of 70%. During their growth, the plants were watered every other day and given Kristallin Blue nutrient solution (16N : 6P : 20K : 3Mg) at 1g/L once a week. At the start of the experiment, the plants were approximately six weeks old. Excised leaves from the plants were then used to feed either *Athalia rosae* or *Pieris rapae* larvae (see below).

Cultures of *A. rosae* were reared in the laboratory on black mustard (*B. nigra*) plants on which the adult females preferred to oviposit. The plants were grown using the same methods as described for cabbage.

Athalia rosae. A culture was started with *A. rosae* pupae provided by Bielefeld University, Germany. The insects were maintained in a climate-controlled room at 22±1 ^{0}C, a photoperiod of 16L:8D and a r.h. of 50%. The adults were maintained in mesh cages of 35x35x60 cm (Vermandel, The Netherlands), fed honey and water and were provided with potted *B. nigra* plants for oviposition. Plants with eggs were transferred to new cages and the larvae were reared on these plants until they pupated in the soil. Emerged adults were added to the cages with adult *A. rosae*.

Pieris rapae. First and second instar *P. rapae* larvae were provided by the Lab. of Entomology at Wageningen University, where a culture of this species has been reared on cultivated cabbage (*Brassica oleracea* var. *gemmifera*, Cv. Cyrus) for many generations. Wild *P. rapae*, collected from the surroundings of Wageningen, are regularly added to the culture.

Podisus maculiventris. The colony of the predator *P. maculiventris* was established from eggs supplied by the Agricultural Research Service, United States Department of Agriculture (USDA-ARS-CMAVE) at Gainsville (FL, USA). Insects were maintained at the NIOO in a climate chamber at 16±1 ^{0}C, a photoperiod of 16L:8D and a r.h of 70%. Immature stink bugs were reared in Petri dishes (5 cm diameter for L1-L3 nymphs and 14 cm diameter for L4-L5 nymphs) provided with water-saturated cotton balls. L1 nymphs are not predaceous and instead feed exclusively on water. The remaining four (L2-L5) instars were fed a mixed diet of various stages of lepidopteran larvae (*Pieris brassicae*, *Spodoptera littoralis* and *Mamestra brassicae*) and cocoons of the parasitic wasp *Cotesia glomerata* that were also maintained in culture. *Pieris brassicae* and *M. brassicae* were reared on Brussels sprouts (*Brassicae oleracea* var. gemmifera cv. Cyrus), whereas *C. glomerata* was reared on *P. brassicae* caterpillars feeding on the food plant. *Mamestra brassicae* and *S. littoralis* were reared on artificial diet as described in Shorey and Hale (1965).

Adult stink bugs were kept in mesh cages of 32x32x32 cm (BugDorm, Taiwan) and fed on the same mixed diet as the nymphs. Water was provided in small Petri dishes with humidified cotton balls. Folded paper towels were placed in the cage to provide shelter and oviposition sites. Twice a week the towels were checked for egg batches, which were then carefully removed and kept in small Petri dishes until hatching.

Performance study

Prey preparation. Different instar stages of A. rosae larvae were transferred to plastic containers and provided with leaves of one of the three different plant populations for four days. This time span is long enough to ascertain that the GS content in the larvae resembles that of their current food plant (Müller and Wittstock 2005). Each day fresh leaves were provided. After the prey conditioning period the larvae were fed to the stink bugs. New batches of prey feeding on the different plant populations were prepared daily. The same protocol was followed for the *P. rapae* larvae.

Bioassay. The experiment was conducted in a climate room at 22±1 ^{0}C, a photoperiod of 16L:8D and a r.h. of 50%. Immature development of *P. maculiventris* was recorded when feeding on two different prey species whose larvae differ in their ability to sequester GS from their host plant: *A. rosae* (sequestering) and *P. rapae* (non-sequestering). In addition, we varied food plant chemistry by rearing both prey species on wild cabbage plants originating from three different populations that are known to vary qualitatively and quantitatively in GS chemistry. This resulted in six different treatments: two prey species reared on three different food plants. Fifty stink bugs were assigned to each treatment.

During the experiment, 3-5 stink bug individuals were kept per Petri dish (due to the varying availability of newly hatched nymphs). The first instar of the predatory bug does not consume prey and only imbibes water, therefore, these were only provided with water-saturated cotton balls. From the second instar until adult egression, the stink bugs were fed *ad libitum* with prey items from one of the six diets. The amount of provided prey items and their developmental stage depended on the amount of stink bugs in a dish, whether bug nymphs were moulting, and the size of the prey items. This meant that on average 1-3 fresh prey items per bug were provided daily. Second instar bug nymphs were provided with second instar prey items. Larger, later instars of the bug nymphs received larger prey. Humidified cotton balls were provided as a water source. For each treatment, 50 newly moulted L2 nymphs were randomly selected from several egg batches. Every day dead prey items were removed and a fresh supply of prey was offered to ensure that excessive food was always available. Nymphal survival (to adult egression), development time from the second instar to adult and fresh adult body mass of the stink bugs were recorded.

Statistical Analyses

Adult body weight and development time were analysed using a mixed model with plant population, prey species, sex and their interaction terms as fixed factors and Petri dish (=replicate) as a random factor. If the three way interaction term was significant, the data were also analysed for males and females separately to reveal more clear patterns. Post-hoc Tukey Kramer multiple comparison tests were conducted to reveal differences among means. The data on development time were log-transformed to meet assumptions of normality and homoscedasticity. The survival data were analysed with a Kruskal-Wallis one-way ANOVA test. The performance data were analysed with SAS 9.3.

Results

Survival

There was no significant difference in survival of *P. maculiventris* between prey species or plant populations (Kruskal-Wallis, H_5=3.809, x^2=0.189). Survival ranged between 88% and 100% (Fig. 1).

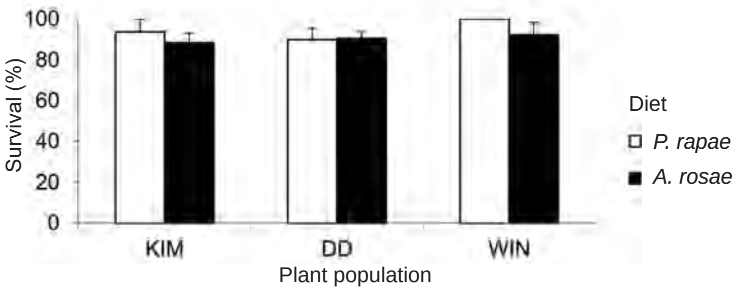

Figure 1 Survival (mean ± SE) of *P. maculiventris* per plant population and prey species (n=50). The white bars represent *P. rapae* as prey species and the black bars *A. rosae*.

Development time

The effect of prey species on development time of the stink bugs was similar for females and males ($F_{2, 211}$=0.45, P=0.641). The development time of the stink bugs was marginally affected by the interaction term of plant population and prey species ($F_{2, 56.5}$=3.14, P=0.051). For DD and KIM, development was fastest on the *P. rapae* prey items, but for WIN development was fastest on *A. rosae* prey items. Both sexes developed slowest on *A. rosae* larvae that had fed on leaves from the DD population (Fig. 2). However, these trends were not statistically significant.

Adult body mass

Adult body mass of *P. maculiventris* was significantly affected by the interaction between plant population, prey species and sex ($F_{2, 241}$=4.69, P=0.01). Therefore, the data were analysed separately for males and females to improve interpretation of the results. In female predators, the interaction between prey species and plant population significantly affected the body mass ($F_{2, 48.8}$=5.15, P=0.009, Fig. 3A). The effect of prey species differed with plant population and no consistent effect of prey species could be discerned. In males, there was a significant effect of prey species on body mass ($F_{1, 49.5}$=4.37, P=0.04) with larger stink bugs produced when reared on *P. rapae* than on *A. rosae* and this effect was similar on all plant populations (interaction plant population and prey species: $F_{2, 49.1}$=0.11, P=0.89). The main effect of plant population on body was not significant ($F_{2, 49.1}$=2.14, P=0.13, Fig. 3B).

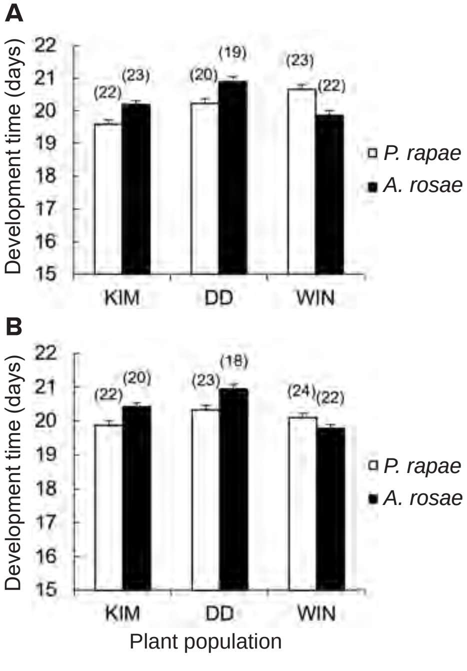

Figure 2 Female (A) and male (B) development time (mean ± SE) of *P. maculiventris* per plant population. The white bars represent *P. rapae* as prey species and the black bars *A. rosae*. Note that the y-axis starts at 15 days. The effect of plant population and prey species was not statistically significant (see text for statistics) and similar for males and females. The number of individuals (n) are shown within parentheses.

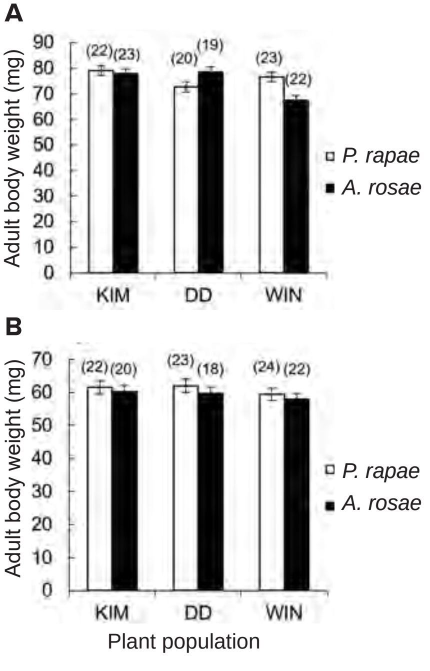

Figure 3 Female (A) and male (B) adult body mass (mean ± SE) of *P. maculiventris* per plant population. The white bars represent *P. rapae* as prey species and the black bars *A. rosae*. Bars with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means with α = 0.05). The number of individuals (n) are shown within parentheses.

Discussion

In this study we found that neither the prey species nor plant population affected nymph to adult survival of *P. maculiventris*, revealing that both direct (prey) and indirect (plant) diets for the predator were highly suitable for their development. However, differences in both the prey species and plant population did affect other performance-related parameters in *P. maculiventris*. The effects of prey species and plant population were sex specific. Males were heavier when reared on *P. rapae* than on *A. rosae* and this was similar on all plant populations. For females the effects of prey species diffed with plant populations and no clear pattern was discerned. The effects of prey species on development time of the stink bugs was similar for males and females. Stink bugs developed faster on *P. rapae* than on *A. rosae* that had been feeding on KIM and DD plants, whereas this was reversed on WIN plants. These results clearly illustrate the complexity in trait- and context-dependent interactions involving only a small number of species.

Importantly, our results, particularly with respect to nymph-adult survival, reveal no clear evidence of a significant negative effect of sequestration by *A. rosae* on the performance of *P. maculiventris*. This is emphasized by the fact that the predator is native to North America and has only recently been introduced into Europe as a biological control agent (De Clercq and Degheele 1994; De Clercq et al. 1998). A major component of the study was to ensure that the predator was naïve and had no evolutionary history with either prey species, generating a novel interaction. However, the results do indicate that *A. rosae* was of marginally lower quality than *P. rapae* for the development of *P. maculiventris*. Adult body mass of the male stink bugs was significantly lower when reared on *A. rosae* in all three plant populations, and development times tended to be slightly shorter on *P. rapae*. However, a major point (and one that needs to be addressed in other lab studies conducted under strictly controlled conditions, and where results are similarly ambiguous) is whether the observed variation is ecologically significant. Given the high suitability of both prey irrespective of the plant population-related diet, the results presented here reveal that *P. maculiventris* performs well on both sequestering and non-sequestering prey. Also, the small difference in prey quality could be the result of sequestered GS in *A. rosae* and potentially other compounds in *P. rapae*, which we did not measure.

Extensive work on GS sequestration by *A. rosae* has been carried out by Müller and colleagues. Larvae of *A. rosae* are able to selectively incorporate and concentrate intact GS from the host plant into their haemolymph (Müller et al. 2001). Consequently, specific GS have been found at much higher concentrations in the haemolymph than in the plant leaves (Müller et al. 2001; Abdalsamee and Müller 2012). For example, a study on the performance and sequestration by *A. rosae* on wild cabbage populations from KIM and WIN found that the composition of GS in larval haemolymph differed significantly from GS in the host plant tissues (Abdalsamee and Müller 2012). Larvae from the WIN population where characterized by high concentrations of aliphatic GS, whereas larvae from KIM populations had much smaller overall concentrations and were characterized primarily by indole GS (Abdalsamee and Müller 2012). Aliphatic GS were sequestered in relatively high amounts from both plant populations, but indole GS only from KIM plants. Differences in larval GS profiles may explain why growth and body mass of female stink bugs on WIN was more negatively affected when reared on the sequestering *A. rosae* than on the non-sequestering *P. rapae*. However, the same negative effect was not found on the male stink bugs, suggesting that the consequences of sequestration are negligible or else 'finely-tuned'.

Several studies have investigated whether sequestration of plant allelochemicals by *A. rosae* is an effective defence mechanism against attack by natural enemies such as invertebrate and vertebrate predators. Previous work has focused mostly on the deterrent effects of easy bleeding on predator foraging behaviour and susceptibility of the sawfly larvae to attack. It has been shown that the (extracted) haemolymph acts as a feeding deterrent on the ant *Myrmica rubra* (Müller et al. 2002; Opitz et al. 2010). When separate GS compounds were presented to ants in the same concentration as found in the sawfly's haemolymph, they were also deterred but to a lesser extent. This suggests that other compounds in addition to GS may play a role in the defensive arsenal of *A. rosae* (Müller et al. 2002). In a more natural setting, haemolymph released by easy bleeding was found to act as a defence against the predatory wasp *Vespula vulgaris* (Müller and Brakefield 2003). The behaviour of another predatory wasp, *Polistes dominulus*, was dependent on the host plant that the larvae of *A. rosae* had been feeding on. The wasp rejected significantly more larvae that had been reared on *Barbarea stricta* than on *Sinapis alba* (Müller and Arand 2007). Unattractiveness of the sawfly larvae was also demonstrated with a vertebrate predator, the lizard *Anolis carolinensis*; however, it was concluded that prey movement (which was less than other prey items) and warning colour of the larvae may also be important determinants in the foraging of predators that rely primarily on visual cues (Vlieger et al. 2004). The above studies provide ample evidence that easy bleeding does deter attack from generalist predators. However, little attention has been paid to how sequestration, another defensive mechanism, affects the development of natural enemies in no-choice assays. Ours is the first study to examine the complete development of a naïve predator.

A previous study reported that GS sequestered by larvae of *A. rosae* were found in body tissues of *P. maculiventris* that had consumed them, but only immediately after feeding. After 24 hours, however, no GS were found, suggesting that the stink bugs can effectively detoxify and/or excrete these compounds (Boevé and Müller 2005). This means that GS are transferred from *A. rosae* to this predatory species. The absence of a negative effect of sequestration on the development of *P. maculiventris* may be due to the fact that *A. rosae* larvae do not produce their own MYS, which, as explained earlier, are necessary for the production of toxic breakdown products. This contrasts with findings in other sequestering Brassicaceous-feeding herbivores that are considered to be highly toxic to some natural enemies. For example, the aphid species *Brevicoryne brassicae* feeds on plant phloem, which prevents cell disruption but also enables the uptake of intact GS (Winde and Wittstock 2011). Because this species also synthesizes its own MYS, it is able to use the GS-MYS system for its own defence (Jones et al. 2001). MYS from the plant are found in *A. rosae* larvae, but they are stored in the gut, whereas the GS are stored in the haemolymph, and thus both do not physically come in contact with one another (Müller 2009). It has been speculated that after severe attack with concomitant physical damage, the GS and MYS do come into contact with each other, upon which toxic hydrolysis by-products are formed (Müller 2009). This is not the case with the process of easy bleeding in *A. rosae*, when only GS in the haemolymph act as a defence. Bleeding behaviour was not observed when *A. rosae* larvae were attacked by *P. maculiventris* (see also Boevé and Müller 2005). The mechanism is apparently prevented because the feeding stylet of the bug pierces the integument of the larvae quickly; the process may differ with predators that possess biting mouthparts, such as ants and wasps (Boevé and Müller 2005).

It is known that *P. maculiventris* consumes low numbers of prey in the field, about one prey item every other day (O'Neil 1988), whereas in the current experiment prey larvae were refreshed daily. It is possible, on the basis of the findings in previous studies, that a negative effect of GS on the performance of *P. maculiventris* may only occur if prey availability is reduced below a critical threshold (Weiser and Stamp 1998). During the experiment we observed that larvae of *A. rosae* did not consume its prey piecemeal, but instead that some tissues remained, a finding also observed by Boevé and Müller (2005). If the stink bugs had been forced by prey limitation, then deleterious effects of GS sequestration may have been found.

In summary, this study reports that the development, and in particular survival, of a naïve generalist predator, *P. maculiventris*, is only marginally affected by allelochemicals sequestered by larvae of the turnip sawfly, *A. rosae*. This prey species was of similar suitability and marginally lower quality then caterpillars of *P. rapae* of approximately the same size during each larval instar. Although *P. maculiventris* bypasses the feeding deterrent effect of easy bleeding, it is nonetheless challenged with sequestered GS in the haemolymph of *A. rosae*. However, the separation of the GS and MYS components in the larvae of *A. rosae* possibly renders them as being palatable to natural enemies that are able to circumvent or suppress a rapid bleeding response. One possibility is that the predatory bug is able to feed selectively on tissues of *A. rosae* and thus able to avoid the more toxic tissues. Interestingly, both *A. rosae* (that does not produce the toxic breakdown products of GS and MYS) and *B. brassicae* (that does) both harbour few, if any specialist enemies. No parasitoids are known to attack *A. rosae* and only a single endoparasitoid, *Diaeretiella rapae*, attacks *B. brassicae* in the field. Given that some sawflies and most aphids are attacked by many parasitoids (Star***ý* 1970;** Price 1972), this suggests that both modes of sequestration may still provide defensive functions against certain types of ‘intimate’ natural enemies, such as endoparasitoids.

It is clear that sequestration of GS by *A. rosae* benefits its defence, not only against endoparasitoids but also other natural enemies, as mentioned earlier. However, we conclude that sequestration of GS by *A. rosae* is largely ineffective as a defensive mechanism against *P. maculiventris*. We suggest that sequestration of glucosinolates by *A. rosae* functions not only as a defensive mechanism against some predators, but may also be an alternative way of harmlessly dealing with plant allelochemicals.

Chapter 4

Interactions between a belowground herbivore and primary and secondary root metabolites in wild cabbage

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Published in: *Journal of Chemical Ecology* 41: 696-707 (2015)

Abstract

Plants are attacked by both above- and belowground herbivores. Toxic secondary compounds are part of the chemical defence arsenal of plants against a range of antagonists, and are subject to genetic variation. Plants also produce primary metabolites (amino acids, nutrients, sugars) that function as essential compounds for growth and survival. Wild cabbage populations growing on the Dorset coast of the UK exhibit genetically different chemical defence profiles, even though they are located within a few kilometres of each other. As in other Brassicaceae, the defensive chemicals in wild cabbages constitute, among others, secondary metabolites called glucosinolates. Here we used five Dorset populations of wild cabbage to study the effect of belowground herbivory by the cabbage root fly on primary and secondary chemistry and whether differences in chemistry affected the performance of the belowground herbivore. There were significant differences in total root concentrations and chemical profiles of glucosinolates, amino acids, and sugars among the five wild cabbage populations. Glucosinolate concentrations not only differed between the populations, but were also affected by root fly herbivory. Amino acid and sugar concentrations also differed between the populations, but were not affected by root fly herbivory. Overall, population-related differences in plant chemistry were more pronounced for the glucosinolates than for the amino acids and the sugars. The performance of the root herbivore did not differ among the populations tested. Survival of the root fly was low (<40%) suggesting that other belowground factors may override potential differences in effects related to primary and secondary chemistry.

Introduction

The study of plant-insect interactions has is a foundation for our understanding of evolutionary and community ecology (Schoonhoven et al. 2005). Early studies on plant-insect interactions focused primarily on the aboveground compartment, ignoring the fact that plants, through their roots, also interact with the biotic environment belowground. The importance of biotic interactions in the rhizosphere has become apparent in the past two decades (Masters and Brown 1992; Masters et al. 1993; van der Putten et al. 2001). Moreover, the belowground environment has consequences for biotic interactions with aboveground plant tissues and vice versa (Wardle et al. 2004). Plants are attacked by insect herbivores both in the aboveground and belowground domains, often simultaneously. It has been shown that belowground herbivores, by removing root tissues, negatively affect the functioning of roots, for instance through a reduction in the uptake and storage of nutrients, which cascade to other plant tissues thereby affecting the whole plant (Blossey and Hunt-Joshi 2003; van der Putten 2003).

Plants tissues produce both primary and secondary chemical compounds (metabolites) that have different biological functions. Primary metabolites are compounds that plants need in order to grow, develop and reproduce, and include amino acids and sugars (Bidwell 1974; Gibson 2005). Plants also produce toxic secondary metabolites to protect plant organs, especially those that are important for survival and reproduction, against herbivores and pathogens. Secondary metabolites are also used to complete with other plants, to attract pollinators and seed dispersers, to mitigate symbiotic interactions and to protect against UV-light or other physical stress (Wink 1999). The balance between concentrations of secondary and primary metabolites is an important determinant of food plant quality for insect herbivores (Awmack and Leather 2002; Scriber and Slansky 1981). Plant primary metabolites provide essential nutrients for insect development, whereas secondary metabolites can be repellent and/or toxic for many insects and thus interfere with insect behaviour and physiology (Schoonhoven et al. 2005; Scriber and Slansky 1981). However, for many co-evolved specialist herbivores host-derived secondary metabolites function as feeding or oviposition stimulants (Schoonhoven et al. 2005).

Plant secondary chemistry is phylogenetically conserved and genetic variation among populations is often maintained over different scales of space and time (Agrawal et al. 2012; Berenbaum and Zangerl 1991; Hartmann 1996; Hoy et al. 1998; van Geem et al. 2013). Various studies have shown that the concentrations and specific compounds of secondary metabolites differ between species within a plant family, individuals and populations within a species, and even plant structures of individual plants (Fahey et al. 2001; Fordyce and Malcolm 2000; Gols et al. 2008b; Häring et al. 2007; Hartmann 1996). So far, studies on the evolution of diversity in secondary metabolites driven by insect herbivores have focused on aboveground plant tissues. Little is known about variation in defence chemistry in the rhizosphere and effects of defensive chemistry on belowground herbivores. Moreover, intra-specific variation in primary chemistry has been virtually ignored.

Wild cabbage (*Brassica oleracea*) plants grow naturally along the Atlantic coasts of north-western Europe and belong to the large family Brassicaceae. Plant species within this family all produce glucosinolates (hereafter GS), inducible secondary plant metabolites that play an important role in mediating plant-insect interactions (Gols et al. 2009, Hopkins et al. 2009). GS profiles not only differ between populations (Gols et al. 2008b; Mithen et al. 1995; Moyes et al. 2000; Newton et al. 2009; van Geem et al. 2013), but also between individual plants within a population (Mithen et al. 1995) and between different plant organs of individual plants (Bennett and Walsgrove 1994). The variation in defence chemistry profiles makes the English wild cabbage populations a good model system for studying the effect of plant secondary metabolites on insect performance while incorporating genetic variation that is maintained over a limited spatial scale (e.g. 20 km).

Induction of GS has been well studied in aboveground plant tissues (Agrawal et al. 1999; Agrawal 2000; Gols et al. 2008a,b; Harvey et al. 2007,2011; Poelman et al. 2008;) but less so in belowground tissues (Soler et al. 2005; van Dam and Raaijmakers 2006). Moreover, the number of studies investigating the combined effect of primary and secondary metabolites in leaves on insect performance in wild plants are limited (but see Cole 1997; Wurst et al. 2006). Even less is known about variation in primary and secondary chemical profiles in roots, their effect on root insect development, and whether concentrations of these chemicals change in response to belowground herbivory, which is the topic of this study.

The main aim of our study was to determine whether the chemical profiles of primary and secondary metabolites in the roots differed between the wild cabbage populations in response to belowground herbivory. We were also interested if there was a link between root fly performance and root chemistry. In a greenhouse experiment, we grew plants from seeds collected from five naturally growing populations in Dorset, England that differ in foliar GS chemistry (Gols et al. 2008b; Newton et al. 2010). We compared development of a specialist herbivore, the cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae) on these plants. In addition to root chemistry, we also measured root biomass and insect performance variables. Given that previous studies have shown that there is constitutive and inducible variation in GS chemistry in leaf tissues of the cabbage populations, we hypothesize that 1) this variation is also present in the roots, and that 2) the performance of the root flies will differ when grown on the different populations, as has been demonstrated with aboveground insects.

Methods and Materials

Plants

Wild cabbage (*Brassica oleracea*) seeds were collected from five populations that are located on the southern coast of the UK, in the Dorset area near Swanage (Fig. 1). The seeds were collected from multiple plant individuals per population. The five populations are Durdle Door (DD; 50^{0}62′N, 2^{0}27′W), Kimmeridge (KIM; 50^{0}35′N, 2^{0}03′W), St. Aldhelms Head (SAH; 50^{0}69′N, 2^{0}05′W), Winspit (WIN; 50^{0}34′N, 2^{0}02′W) and Old Harry (OH; 50^{0}38′N, 1^{0}55′W). Each population has a unique microhabitat; WIN is sheltered from the prevailing southwest wind, OH and DD are partially wind-exposed, whereas KIM and SAH plants are exposed to the wind because they grow on top of the cliffs facing south to southwest. Differences in microhabitat affect the herbivore pressure at each location, since highly exposed locations are likely to be less accessible to herbivores than secluded ones.

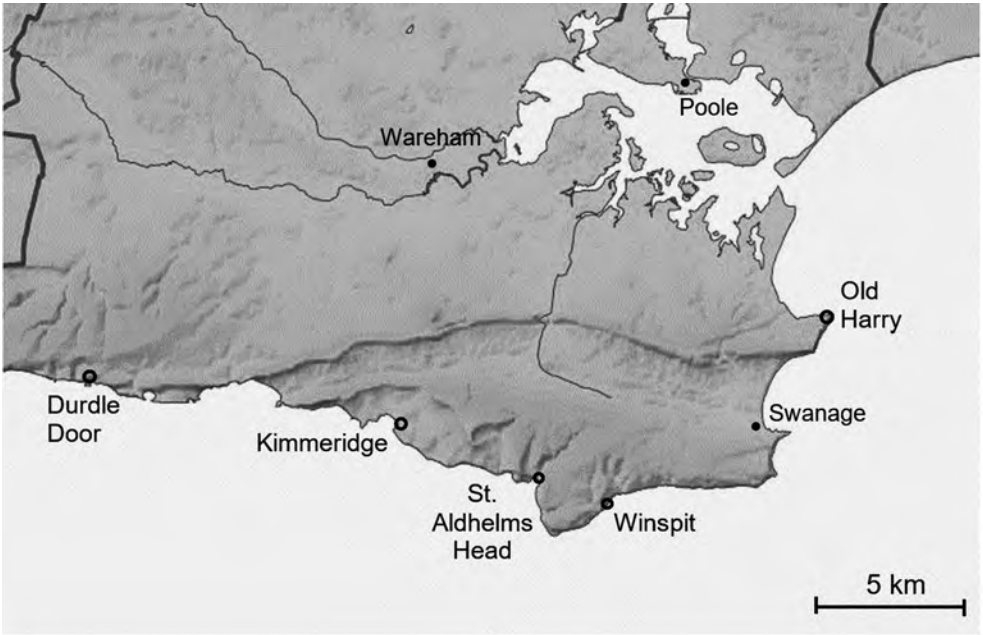


Figure 1 Locations where the five wild cabbage (*Brassica oleracea*) populations (open dots) are located in Dorset, UK

The seeds were germinated in germination soil (Lentse Stekgrond, Lent, The Netherlands) and after one week seedlings were transplanted into individual 2-L pots containing a soil mixture of 11% clay, 69% peat and 20% pumice. The plants were grown in a glasshouse at 21±1^{0}C (day) and 16±1^{0}C (night) with 70% r.h. and a L16:D8 light regime. They were watered every other day and given extra nutrients twice a week using half-strength Hoagland solution (Hoagland and Arnon 1950). The plants were 8 weeks old when they were used in the experiments.

Insects

The cabbage root fly (*Delia radicum*) is a common pest species in agriculture and a specialist on plant species from the Brassicaceae family. Female flies lay their eggs near the stem-root interface and newly hatched larvae crawl into the soil and burrow into the upper roots. The larvae feed on the root tissue and can have severe negative effects on the growth of their host plant.

The root fly culture was established at the NIOO in Wageningen from root fly pupae that were provided by the University of Rennes, France. That colony was started in September 2009 with flies collected in the field (Le Rheu, Brittany, France, 48°07016″ N, 01°47041″ W). The root flies were reared in a climate room (21±1^{0}C, 50-70% r.h., L16:D8) on a diet of sugar, milk powder and nutritional yeast (1:1:1) and maintained on swedes or turnips. Water was provided through humidified cotton balls. To obtain larvae for the experiment, adult root flies were offered a fresh piece of turnip placed in a 9 cm Petri dish with moist filter paper as an oviposit site. After one day the eggs were collected and kept on moist filter paper until they developed into first instar larvae which were used to inoculate the plants.

Experimental design

From each plant population 20 plants were assigned to the root fly induction (= belowground herbivory) treatment and 10 plants to the control (= no herbivory) treatment. Newly hatched root fly larvae, 3 larvae per plant, were carefully transferred to the stem/root interface of plants with a small brush. After transfer, the larvae were observed to confirm that they crawled into the soil. Two weeks later, every plant was put into an individual sleeve cage (48 x 71 cm, 104 x 94 mesh; Bugdorm, MegaView Science, Taiwan) to contain emerging adult root flies.

Emergence of adults was recorded three times a day and their development time was determined as the number of days between egg hatching and adult eclosion. Survival was calculated as the number of flies per plant that emerged from the three fly larvae that were initially introduced. Adults were killed immediately after eclosion by freezing at -20 ^{0}C. Their dry body mass was obtained by drying them in an oven at 70^{0}C for three days. After all root flies emerged, the plants were harvested to record root biomass (clean roots were weighed prior to storage) and the roots of the plants were sampled to analyse glucosinolates, amino acids and sugars (described below).

Chemical root analysis

The roots of all 30 plants per population, 150 in total, were removed from the soil, washed, placed in paper bags and stored at -20 °C until further processing. Roots were freeze-dried for 4 days. Dried roots then were cut up in small pieces and a representative sample (parts from both thick and thin roots) was selected for further analysis. These samples were ground to powder with a grinding machine (Retch, type MM 301).

For the methanol extraction of GS, sugars and amino acids, approximately 50 mg of the ground root was weighed in a 2 ml microfuge tube. Samples were suspended in 1 ml of 70% MeOH and vortexed before boiling in a water bath for 5 min. Samples were transferred to an ultrasonic bath for 15 min and centrifuged for 10 min at 10,000 rpm to obtain clear supernatant solutions that were transferred to clean microfuge tubes. The extraction was repeated, and the second extract for each sample was pooled with the first.

The mass of each sample was adjusted to be equal (+/- 0.01 g) to the average mass of a standard 2 ml aliquot of 70% MeOH.

Glucosinolate analysis

One ml of the extract was added to a DEAE-Sephadex A25 column, followed by two additions of 1 ml of 70% MeOH, one addition of 1 ml of MilliQ water, and two additions of 1 ml of 20 mM sodium acetate buffer (pH = 5.5). Then 20 µl of sulfatase (Sigma type H-1 from Helix pomata) in aqueous solution was added to each column followed by 50 µl of 20 mM sodium acetate buffer. The columns were placed over vials, covered with aluminium foil, and left to stand overnight. The next day, the desulfoglucosinolates were eluted from each column by washing with 1 ml of MilliQ water twice, and the samples were freeze dried. Each freeze dried sample was redissolved in 500 µl MilliQ water and pressed through a 0.2 µm nylon syringe filter into a HPLC vial.

High-performance liquid chromatography (HPLC) was used to measure concentrations of GS, sugars and amino acids. The GS analyses were performed with an Alltima C18 column (150 x 4.6 mm) using 50 µL injection volume. The column was kept at a temperature of 40°C and the flow rate was 0.75 ml/min. Glucosinolates were detected with a UV diode array at a wavelength of 229 nm. Sinigrin in five different concentrations (63 µM – 625 µM) was used as an external standard for the quantification of the GS. Individual GS were identified based on their retention times and UV spectra compared to those of the standards (EC Community Bureau of Reference, Brussels, Belgium, BCR-367R). Final concentration (nMoles/mg) were calculated by correcting for the volume and dry mass of the extract and original tissue.

Soluble sugar analysis

Ten µl of the stock extract were mixed with 990 µl MilliQ water in an HPLC vial. Samples were analysed on the ion-exchange HPLC with a CarboPac PA1 main column (2 x 250 mm) and a CarboPac PA1 guard column (2 x 50 mm) using 5 µl injection volume. The columns were kept at a temperature of 20°C and the flow rate was 0.25 ml/min. The standard curves of 11 sugars (2.5-10 ppm) were used as reference. Final concentrations (µg/mg) of sugars in the roots were calculated by correcting for the volume and dry mass of the extract and the original tissue.

Amino acid analysis

Twenty µl of the stock extract were mixed with 980 µl MilliQ water in an HPLC vial. Samples were analysed on the ion-exchange HPLC with an AminoPac PA10 main column (2 x 250 mm) and an AminoPac PA10 guard column (2 x 50 mm) using 25 µl injection volume. The column was kept at a temperature of 30°C and the flow rate was 0.25 ml/min. The standard curves of 20 amino acids (1-8 µM) were used as reference. Final concentrations (nMoles/mg) of amino acids in the roots were calculated by correcting for the volume and dry mass of the extract and the original tissue.

Statistical analysis

All univariate analyses were performed using SPSS version 22 (IBM SPSS Statistics) and the multivariate analyses with Canoco version 5.03 (ter Braak and Šmilauer, The Netherlands).

Total metabolite levels and the root biomass data were analysed using two-way ANOVA with plant population and root fly treatment, as well as their interaction, as fixed factors. When needed, data were log- or square-root-transformed to meet assumptions of normality and homogeneity of variance. Post-hoc Tukey multiple comparison tests were performed when the ANOVA models were significant.

Chemical profiles of the roots of all plant populations were analysed using multivariate principal component analysis (PCA) and redundancy analysis (RDA) to test for differences between plant populations and treatments. RDA is a linear method of canonical ordination also described as a direct gradient analysis technique that summarizes linear relationships between response variables (here concentrations of chemicals) that are explained by a set of variables (here populations and treatments) (Lepš and Šmilauer 2003).

Concentrations of chemicals were log-transformed and mean-centered. Correlations between the chemical data and the insect performance and root biomass data were tested with RDA in Canoco. Adult body mass and development time were tested separately for the female and male root flies.

The root fly performance parameters development time and adult body mass were analysed using a linear mixed model with plant population and sex as fixed factors. Plant ID was used as a random factor to deal with the fact that the three data points (three root flies) per plant were not independent. Insect survival data were analysed with binary logistic regression.

Results

Glucosinolates

Thirteen different GS were present in root tissues of all five plant populations (Table 1). Based on their amino acid precursors, GS can be classified into three classes: aliphatic, indole and aromatic (Halkier and Gershenzon 2006). Eight of the GS belonged to the aliphatic, four to the indole and one to the aromatic GS class (see Table 1).

The effect of belowground herbivory on total GS concentration in the roots was dependent on the plant population tested (plant population: $F_{4,136}$=45.1, P<0.001; treatment: $F_{1,136}$=0.261, P=0.610; interaction: $F_{4,136}$=3.59, P=0.008). Belowground herbivory increased the total GS concentration in KIM roots, but not in the roots of the other populations (Fig. 2A). Total GS concentrations tended to be higher in DD, SAH and WIN than in KIM and OH plants.

Analysis of total concentrations of the three GS classes separately showed an interaction effect between population and root fly treatment for the indole GS ($F_{4,136}$=2.57, P=0.041; Fig. 2B), and a population effect for the aliphatic GS ($F_{4,136}$=50.093, P≤0.001; Fig. 2C) and the aromatic GS ($F_{4,135}$=16.727, P≤0.001; Fig. 2D). Although the effect of belowground herbivory on the indole GS concentrations in the roots significantly varied between the populations, no plant population showed a significant increase or reduction of indole GS. Similar to concentrations of total GS, the concentrations of aliphatic GS were highest in DD, SAH and WIN and lower in KIM and OH. The only aromatic GS, gluconasturtiin, had the highest concentrations in DD and lowest in OH.

Table 1 Mean concentrations (±se) of individual gs (μmol/g), sugars (mg/g) and aa (μmol/g) of control plants and induced plants for the five wild cabbage populations.

	Wild cabbage population									
	DD		**KIM**		**SAH**		**WIN**		**OH**	
Compound	control	induced	control	induced	control	induced	control	induced	control	induced
Glucosinolates										
Aliphatic GS										
Glucoalyssin	0.01 ± 0.01	0.03 ± 0.01	-	0.01 ± 0.00	0.07 ± 0.01	0.01 ± 0.01	0.12 ± 0.03	0.08 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
Glucobrassicanapin	0.11 ± 0.05	0.08 ± 0.05	-	0.01 ± 0.00	0.06 ± 0.04	0.09 ± 0.02	0.14 ± 0.06	0.11 ± 0.02	-	0.02 ± 0.01
Glucoerucin	4.91 ± 0.72	4.36 ± 0.65	1.74 ± 0.35	1.82 ± 0.39	8.60 ± 1.02	5.10 ± 0.58	4.14 ± 1.45	7.22 ± 1.25	7.22 ± 1.62	5.70 ± 0.90
Glucoiberin	0.05 ± 0.05	0.33 ± 0.11	0.82 ± 0.09	0.94 ± 0.09	0.25 ± 0.10	0.35 ± 0.08	0.15 ± 0.15	0.26 ± 0.08	0.53 ± 0.17	0.40 ± 0.09
Gluconapin	2.37 ± 0.40	3.66 ± 0.57	0.75 ± 0.22	1.55 ± 0.24	36.96 ± 6.29	39.25 ± 3.81	42.69 ± 7.53	39.35 ± 3.71	10.40 ± 3.14	8.95 ± 1.72
Glucoraphanin	4.23 ± 0.34	3.72 ± 0.31	1.72 ± 0.20	1.57 ± 0.17	4.29 ± 0.31	2.97 ± 0.28	3.31 ± 0.61	3.18 ± 0.30	4.02 ± 0.32	2.95 ± 0.29
Progoitrin	62.94 ± 5.71	46.01 ± 5.60	7.56 ± 1.72	8.96 ± 1.45	10.19 ± 2.20	9.92 ± 1.30	10.84 ± 1.33	8.40 ± 0.66	10.70 ± 2.59	9.48 ± 1.63
Sinigrin	0.95 ± 0.86	4.54 ± 1.33	6.60 ± 1.00	9.27 ± 0.81	3.63 ± 1.46	8.08 ± 2.24	1.08 ± 0.85	5.00 ± 1.24	5.12 ± 1.41	5.01 ± 0.98
Indole GS										
4-methoxyglucobrassicin	1.84 ± 0.40	0.84 ± 0.14	0.82 ± 0.15	0.90 ± 0.14	0.82 ± 0.28	1.22 ± 0.16	1.65 ± 0.26	1.38 ± 0.18	1.55 ± 0.29	1.24 ± 0.17
4-hydroxyglucobrassicin	1.36 ± 0.21	1.14 ± 0.23	0.42 ± 0.07	2.06 ± 0.35	0.32 ± 0.09	0.36 ± 0.05	0.11 ± 0.02	0.22 ± 0.04	0.46 ± 0.10	0.44 ± 0.09
Glucobrassicin	0.98 ± 0.18	0.49 ± 0.07	0.54 ± 0.09	0.59 ± 0.12	0.45 ± 0.14	0.67 ± 0.09	1.09 ± 0.31	0.69 ± 0.08	1.17 ± 0.23	0.75 ± 0.13
Neoglucobrassicin	10.11 ± 2.60	6.29 ± 1.16	4.35 ± 0.69	5.36 ± 1.40	2.12 ± 0.44	4.25 ± 0.88	12.02 ± 2.57	8.34 ± 1.05	3.72 ± 0.83	3.42 ± 0.66

Aromatic GS										
Gluconasturtiin	19.83 ± 1.64	18.27 ± 1.43	7.81 ± 0.81	11.79 ± 0.83	13.73 ± 1.35	11.07 ± 1.04	11.21 ± 1.83	12.43 ± 1.11	9.60 ± 1.16	8.00 ± 0.76
Sugars										
Glucose	1.48 ± 0.15	1.32 ± 0.09	1.40 ± 0.22	1.07 ± 0.10	1.59 ± 0.22	1.34 ± 0.13	1.39 ± 0.17	1.24 ± 0.11	1.28 ± 0.17	1.44 ± 0.11
Fructose	0.46 ± 0.09	0.54 ± 0.06	0.89 ± 0.17	0.60 ± 0.10	1.10 ± 0.25	0.89 ± 0.10	0.91 ± 0.16	0.77 ± 0.18	0.73 ± 0.08	0.98 ± 0.10
Manitol	-	0.01 ± 0.01	0.02 ± 0.02	0.003 ± 0.003	0.003 ± 0.003	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.01
Sorbitol	0.56 ± 0.01	0.60 ± 0.01	0.81 ± 0.08	0.58 ± 0.01	0.76 ± 0.03	0.73 ± 0.02	0.66 ± 0.04	0.69 ± 0.03	0.76 ± 0.01	0.88 ± 0.04
Sucrose	20.44 ± 1.62	20.11 ± 1.49	25.34 ± 2.49	21.36 ± 1.67	30.44 ± 2.74	24.82 ± 1.99	16.27 ± 1.62	18.50 ± 0.79	19.94 ± 3.03	25.21 ± 1.74
Trehalose	0.04 ± 0.02	0.06 ± 0.04	0.02 ± 0.02	0.02 ± 0.01	-	0.07 ± 0.02	0.14 ± 0.05	0.06 ± 0.02	0.09 ± 0.04	0.07 ± 0.02
Amino acids										
Arginine	38.45 ± 2.70	38.56 ± 2.70	46.64 ± 4.19	36.87 ± 2.30	39.21 ± 1.74	39.35 ± 1.88	29.52 ± 4.15	30.72 ± 1.73	43.44 ± 4.11	48.76 ± 2.77
Aspargine	1.58 ± 0.12	1.62 ± 0.21	2.65 ± 0.12	2.25 ± 0.25	1.86 ± 0.15	2.41 ± 0.23	2.28 ± 0.32	2.13 ± 0.11	1.89 ± 0.18	1.94 ± 0.24
Glutamine	2.66 ± 0.30	2.54 ± 0.34	4.14 ± 0.52	3.50 ± 0.39	4.02 ± 0.67	4.49 ± 0.45	4.47 ± 0.82	3.67 ± 0.38	3.35 ± 0.61	3.94 ± 0.84
Histidine	3.36 ± 0.56	4.04 ± 0.66	6.43 ± 0.64	6.41 ± 0.45	3.47 ± 0.83	6.28 ± 1.08	4.87 ± 1.01	5.56 ± 0.54	6.00 ± 0.86	4.84 ± 0.68
Isoleucine	300.7 ± 17.7	296.5 ± 17.6	414.2 ± 21.4	335.3 ± 24.2	450.1 ± 29.0	415.7 ± 25.8	282.9 ± 30.6	284.2 ± 12.3	315.9 ± 39.3	377.9 ± 21.9
Proline	0.60 ± 0.18	1.11 ± 0.25	1.70 ± 0.34	1.84 ± 0.34	0.38 ± 0.19	0.93 ± 0.29	0.26 ± 0.14	0.64 ± 0.26	0.29 ± 0.17	0.44 ± 0.15
Serine	2.13 ± 0.29	2.35 ± 0.27	4.10 ± 0.35	3.95 ± 0.28	2.72 ± 0.46	3.33 ± 0.48	1.63 ± 0.27	2.40 ± 0.29	2.47 ± 0.33	2.58 ± 0.30
Threonine	10.62 ± 0.97	10.08 ± 0.70	11.46 ± 1.67	8.49 ± 0.77	11.48 ± 1.62	10.81 ± 0.95	11.18 ± 1.34	9.23 ± 0.78	10.14 ± 1.32	10.79 ± 0.79
Tyrosine	0.66 ± 0.04	0.67 ± 0.04	0.54 ± 0.07	0.56 ± 0.03	0.52 ± 0.07	0.57 ± 0.04	0.39 ± 0.05	0.38 ± 0.03	0.56 ± 0.03	0.55 ± 0.04

PCA of individual GS concentrations in the roots showed that there was some degree of separation between the GS profiles of the five plant populations (Fig. 3A and B). Four sample clusters are distinguishable from the figure: KIM, DD, OH, and SAH together with WIN. The GS profile of DD was characterized by high concentrations of progoitrin (60% of the total GS concentration), whereas in SAH and WIN gluconapin was the most prominent GS, constituting almost 50% of the total GS concentration. Root tissues of KIM and OH plants did not have one dominant GS, but contained several GS at moderately high concentrations (progoitrin, sinigrin and gluconasturtiin in KIM, and progoitrin, gluconapin and gluconasturtiin in OH). Except for gluconasturtiin which contributed considerably to the GS content (KIM: 24-25%, OH: 17-18%), the dominant class of root GS was formed by aliphatic GS. KIM was separated from the other populations by relatively high concentrations of 4-hydroxyglucobrasicin.

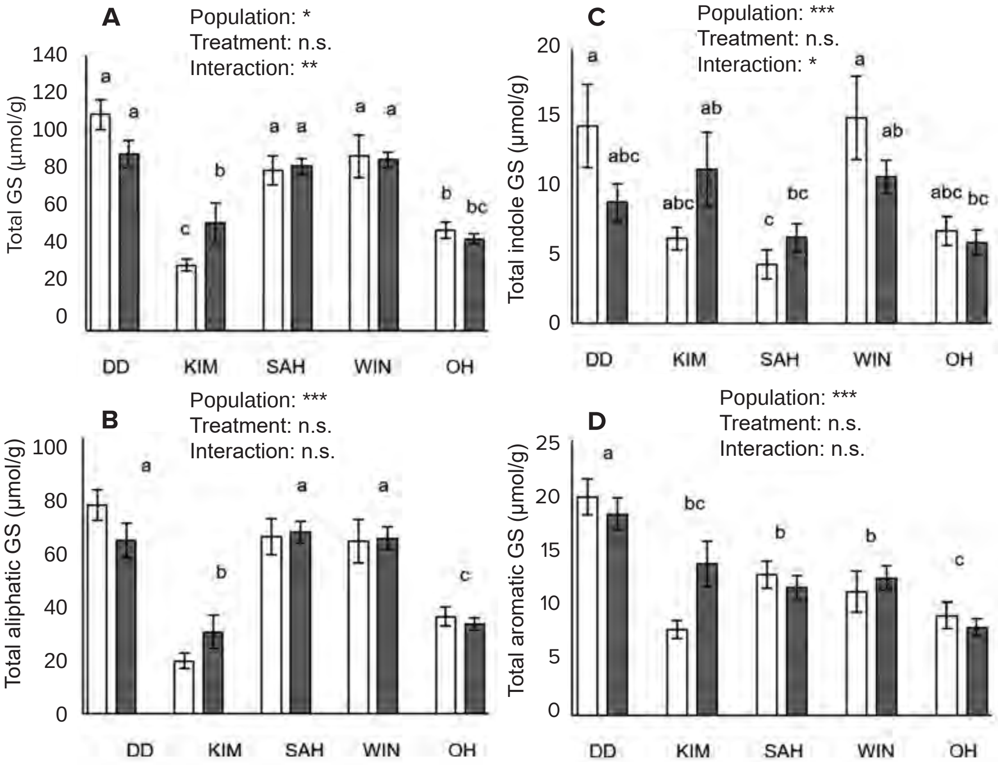


Figure 2 Concentrations (mean ± SE) of total GS (A), indole GS (B) aliphatic GS (C) and aromatic GS (D) in control plants (white bars) and in plants that were damaged by cabbage root fly larval feeding (grey bars) in root tissues of the five wild cabbage populations. *** P≤0.001, ** P≤0.01, * P≤0.05, n.s. = non-significant

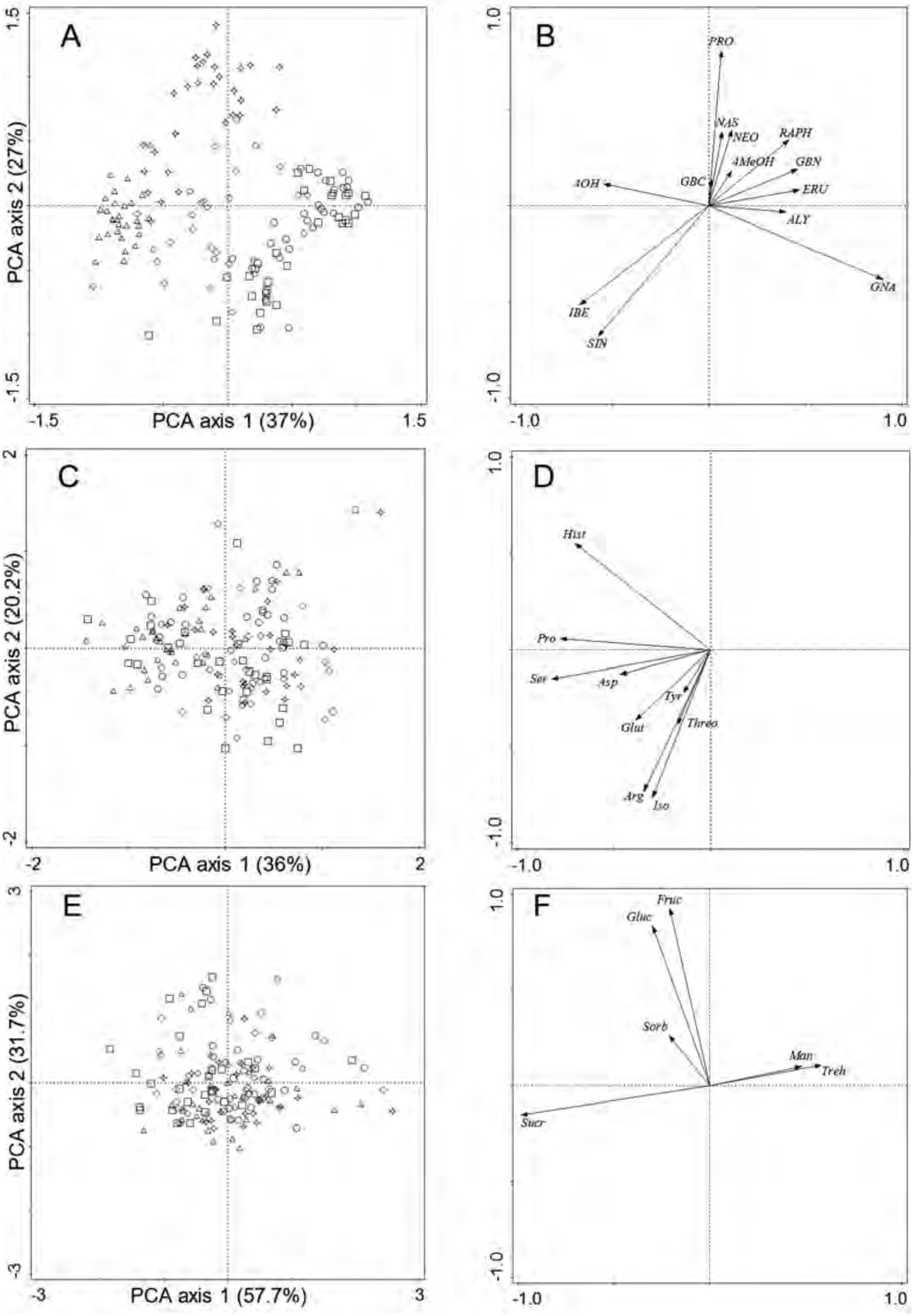
When analysing all individual GS compounds using RDA (figures not shown), the effect of belowground herbivory on the GS profiles of the root tissues depended on the plant population (RDA, population: F=29.6, P=0.001, 45.6% explained variation; treatment: F=2.3, P=0.058, 1.6% explained variation; interaction: F=1.6, P=0.045; 4.5% explained variation). To better interpret the results we did pairwise RDA analyses of control and induced root tissues for each of the five populations.

We found that there was a significant difference between the GS profiles of control and induced root tissues for DD (RDA: F=2.8, P=0.028, 9.4% explained variation), KIM (RDA: F=2.8, P=0.033, 9.5% explained variation) and WIN (RDA: F=3.2, P=0.015, 10.5% explained variation), but not for SAH (RDA: F=1.7, P=0.13) and OH (RDA: F=0.3, P=0.95). The compounds that contributed most to the separation between control and root fly-induced DD samples were sinigrin, 4-methoxyglucobrassicin and neoglucobrassicin. Concentrations of the aliphatic GS sinigrin were higher in induced than in control root tissues (r=0.77), whereas concentrations of the indole GS 4-methoxyglucobrassicin and neoglucobrassicin were higher in control than in induced root tissues (r=0.39 and r=0.35 respectively) (Fig. S1A). Most of the GS compounds were higher in induced than in control root tissues of KIM plants, but especially concentrations of 4-hydroxyglucobrassicin (r=0.76), gluconasturtiin (r=0.46), gluconapin (r=0.4) and sinigrin (r=0.39) (Fig. S1B). In root tissues of the WIN population, concentrations of the aliphatic GS sinigrin and glucoerucin were high in induced (r=0.94 and r=0.51 respectively) and low in control tissues, but the opposite pattern was noted for the indole GS neoglucobrassicin (r=0.3) (Fig. S1C).

Amino acids

Nine different amino acids were detected (Table 1) in root tissues of all five plant populations. Isoleucine was the most dominant amino acid, accounting for more than 80% of the total amino acid concentrations.

The total amino acid concentration in the roots was significantly affected by plant population ($F_{4,136}$=8.107, P<0.001), but not by treatment ($F_{1,136}$=0.016, P=0.90) or by the interaction between plant population and treatment ($F_{4,136}$=1.248. P=0.29). The lowest amino acid concentrations were found in the root tissues of WIN plants and the highest concentrations in SAH plants (Fig. 4A).


A
PCA axis 2 (27%)
PCA axis 1 (37%)
1.5
-1.5
-1.5
1.5
B
1.0
-1.0
-1.0
1.0
PRO
NAS
NEO
RAPH
4MeOH
GBN
GBC
4OH
ERU
ALY
GNA
IBE
SIN
C
PCA axis 2 (20.2%)
PCA axis 1 (36%)
2
-2
-2
2
D
1.0
-1.0
-1.0
1.0
Hist
Pro
Ser
Asp
Tyr
Glut
Threo
Arg
Iso
E
PCA axis 2 (31.7%)
PCA axis 1 (57.7%)
3
-3
-3
3
F
1.0
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Fruc
Gluc
Sorb
Man
Treh
Sucr

Figure 3 PCA ordination plots and loading plots of the glucosinolate profiles (A and B), amino acid profiles (C and D) and sugar profiles (E and F) of the five wild cabbage populations (control and root fly damaged samples both included). The percentage explained variation is given for each axis between parentheses. Populations: DD: star, KIM: triangle, SAH: square, WIN: circle, OH: diamond. Abbreviations: Aliphatic GS: ALY= glucoalyssin, EUR = glucoerucin, GBN = glucobrassicanapin, GNA = gluconapin, PRO = progoitrin, RAPH = glucoraphanin, SIN = sinigrin; Indole GS: 4MeOH = 4-methoxyglucobrassicin; 4OH = 4-hydroxyglucobrassicin, GBC = glucobrassicin, NEO = neoglucobrassicin; Aromatic GS: NAS = gluconasturtiin; Amino acids: Arg = arginine, Asp = asparagine, Glut = glutamine, Hist = histidine, Iso = isoleucine, Pro = proline, Ser = serine, Threo = threonine, Tyr = tyrosine; Sugars: Fruc = fructose, Gluc = glucose, Man = mannitol, Sorb = sorbitol, Sucr = sucrose, Treh = trehalose

The amino acid profiles of all wild cabbage populations and both treatments showed no separation based on explorative analysis from the PCA (Fig. 3C and D, but see Fig. A2A). However, we did find a separation using RDA: there was a significant effect of plant population (RDA: F=5.7, P=0.001, 13.9% explained variation) on the amino acid profiles, but not of treatment (RDA: F=1.5, P=0.17) or the interaction between plant population and treatment (partial RDA: F=1.0, P=0.49) (figures not shown). The root tissues of plants from DD had low concentrations of histidine, glutamine and asparagine, while the root tissues from KIM had high concentration of histidine and also of proline and serine. Root tissues from SAH had high concentrations of isoleucine, and tissues from OH had high concentrations of arginine and low concentrations of proline. The root tissues from WIN had low concentrations of all amino acids.

Sugars

Six different sugars were detected in root tissues of all five plant populations: sucrose, fructose, glucose, sorbitol, manitol and trehalose (Table 1). Sucrose was the dominant compound in all populations, making up almost 90% of the total sugar content.

The total sugar concentration of the roots was significantly affected by plant population (F_4=5.932, P<0.001), but not by treatment (F_1=0.227, P=0.64) nor by the interaction between plant population and treatment (F_4=1.59, P=0.18). Similar to amino acids, the lowest sugar concentrations were found in root tissues of WIN plants and the highest concentrations in root tissues of SAH plants (Fig. 4B).

The sugar profiles of all wild cabbage populations and both treatments showed considerable variation based on explorative analysis from the PCA (Fig. 3E and F, but see Fig. A2B). Using RDA (figures not shown), we found a significant effect of plant population (RDA: F=2.8, P=0.005, 7.3% explained variation) on the sugar root profiles. There was no significant effect of treatment (RDA: F=1.8, P=0.016) or interaction between plant population and treatment (RDA: F=1.4, P=0.17). The root tissues of plants from DD had low concentrations of fructose, sorbitol and sucrose. The root tissues from KIM had low concentrations of glucose and fructose and high concentrations of sucrose. For SAH, the root tissues had high concentrations of fructose and sucrose, while the root tissues of WIN had low concentrations of sucrose. Root tissues from OH had high concentrations of sorbitol, fructose and sucrose.

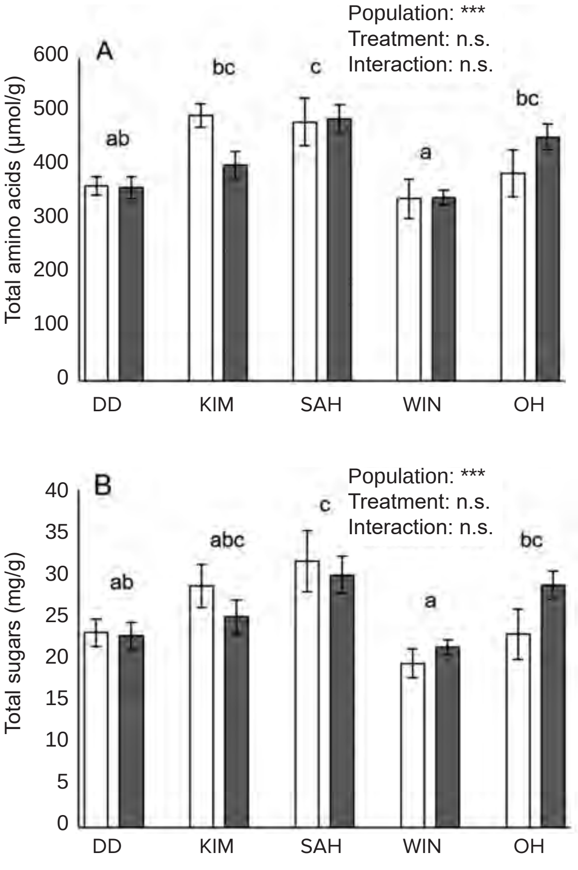


Figure 4 Total concentrations (mean ± SE) of amino acids (A) and sugars (B) in root tissues of control (white bars) and root fly damaged plants (grey bars) for the five wild cabbage populations. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, n.s. = non-significant. Different letters indicate significant differences between populations

Root biomass

Root biomass was similar for all populations (F_4=2.098, P=0.084) and both treatments (F_1=0.007, P=0.94). The effect of the interaction between population and root fly herbivory was also not significant (F_4=1.485, P=0.21).

There was a significant correlation between root biomass and total concentrations of GS, amino acids and sugars (RDA, F=9.1, P=0.002). Root biomass was positively correlated with amino acids (r=0.29) and sugars (r=0.30), and negatively correlated with GS (*r*=-0.1). There was also a significant correlation between root biomass and individual primary and secondary metabolites (RDA, *F*=5.6, *P*=0.001). Root biomass was most positively correlated with the GS sinigrin (*r*=0.27), the amino acids serine and isoleucine (both: *r*=0.21), and negatively with the GS neoglucobrassicin (*r*=-0.71) and 4-methoxyglucobrassicin (*r*=-0.37).

Insect performance

The survival of *D. radicum* did not differ significantly when reared on plants from any of the five populations (logistic regression: X^2=8.75, d.f.=4, *P*=0.07). Survival was generally low with the highest survival (37%) on KIM plants (Table 2). The development time and adult body mass of the root flies also did not differ between the plant populations ($F_{4,\ 39.3}$=1.40, *P*=0.25 and $F_{4,\ 38.6}$=0.41, *P*=0.80 respectively), but did differ between the sexes for adult body mass ($F_{1,41.6}$=5.74, *P*=0.021), with higher body mass for females.

We found a significant correlation between the adult body mass of male root flies and amino acids (RDA, *F*=2.7, *P*=0.037): the body mass was positively correlated with concentrations of histidine (*r*=0.68), asparagine (*r*=0.43), glutamine (*r*=0.4) and serine (*r*=0.25). There were no other significant correlations between insect performance parameters and GS, AA or sugars.

Table 2 Means (±SE) of root fly, *Delia radicum*, survival (%), development time (days) and body mass (mg) for the five wild cabbage populations

	Wild cabbage population				
Performance parameter	DD	KIM	SAH	WIN	OH
Survival	18.33 ±6.15	36.67 ± 7.98	31.67 ± 7.83	21.67 ±8.12	18.33 ± 6.15
Development time	34.64 ± 0.81	35.86 ± 0.6	34.74 ± 0.95	32.92 ±1.03	35.64 ± 0.82
Adult body mass	2.21 ± 0.21	1.93 ± 0.16	2.02 ± 0.16	2.23 ± 0.11	2.13 ± 0.18

Discussion

Our results show that primary (amino acids and sugars) and secondary chemistry (GS) in root tissues differed significantly among the five wild cabbage populations that were compared in this study. This supports our first hypothesis which states that, as has been reported for the leaves, there is also variation in root chemistry among the wild cabbage populations. For the GS, the effect of root fly herbivory differed with plant population. We did not find an interaction between population and root fly treatment for the primary metabolites. Both primary and secondary chemistry can change in response to root fly feeding but these changes are metabolite- and plant population-specific. Interestingly, survival and development of *D. radicum* did not differ, but survival was relatively low (on average 25% of the flies survived to adulthood) on all five *B. oleracea* populations. Thus, we found no support for our second hypothesis in which we state that the performance of the root flies, like that of aboveground herbivores, differs between the cabbage populations.

As reported before for leaf tissues (Gols et al. 2008b; van Geem et al. 2013), the effect of root herbivory on the total and individual concentrations of GS was population-dependent. Interestingly, in belowground root tissues primarily aliphatic GS were induced, whereas in aboveground leaf tissues indole GS very often increase in response to herbivory (Textor and Gershenzon 2009). Gols et al. (2008b) and Harvey et al. (2011) studied the GS profiles of the leaf tissue of KIM, OH and WIN and found that indole GS were primarily induced by feeding damage from specialist herbivores, the small and large cabbage white butterfly *Pieris rapae* and *P. brassicae*. Our results suggest that the type of GS class that is induced in response to herbivory may be plant organ-specific, although the identity of the attacking herbivore and various feeding related traits, (e.g. chewer, phloem-feeder, miner; specialist, generalist) also play a role in the type and strength of plant chemical induction (Bezemer and van Dam 2005, Blossey and Hunt-Joshi 2003).

In a previous study we showed that variation in GS profiles of three populations (KIM, OH and WIN) is more apparent in aboveground than in belowground tissues (van Geem et al. 2013). In this study we did not examine aboveground chemistry, but total GS concentrations in the roots were much higher than in the previous study (van Geem et al. 2013). In both studies, the root GS chemistry of the wild cabbage plants was dominated by aliphatic GS which contributed 55-80% to the total GS content. The aromatic GS, gluconasturtiin which is only found in very low concentrations in leaf tissues of the wild cabbage populations (van Geem et al. 2013), is produced in significant amounts in root tissues (12-25%).

In a study with two *Barbarea vulgaris* chemotypes that were exposed to feeding by *D. radicum*, root GS profiles differed from each other independent of herbivory, but amino acid and sugar profiles did not differ between the chemotypes (van Leur et al. 2008). In our study, both the primary and secondary chemistry differed between the plant populations. However, there are some major differences between the plants used in both studies. Whereas in our study the wild cabbage plants were collected from different populations that are spatially separated, the *B. vulgaris* chemotypes were obtained from a single population (van Leur et al. 2006). This, together with the fact that wild cabbage plants are perennials that live up to 10 years and *B. vulgaris* plants are biennials, suggests that the (a)biotic selection pressures are less diverse for *B. vulgaris* in van Leur et al. (2008) study than in the wild cabbage studied here. This may explain why profiles for primary metabolites of the two *B. vulgaris* chemotypes did not differ significantly. However, in contrast with our results, van Leur et al. (2008) did find an effect of belowground herbivory on primary metabolites: for both amino acids and sugars, total levels were lower in induced plants than in control plants. Hopkins and colleagues (1999) also found that belowground herbivory by *D. radicum* reduced total sugar concentrations in several cabbage cultivars, but this effect differed for individual sugars among cultivars and genotypes. A potentially mitigating factor was that the number of root fly larvae used for inoculation was lower in our study than in the one by Hopkins and colleagues (1999). Although we found an induction effect for GS, perhaps the number of root fly larvae was too low to stimulate dramatic changes in primary chemistry.

The performance of the root flies was not significantly different across the wild cabbage populations, despite the different primary and secondary chemical profiles of the plants. It may be that *D. radicum*, a specialist herbivore that uses GS as oviposition stimulants (Griffiths et al. 2001), is well adapted to a wide range of GS compounds and concentrations and thus is not negatively affected by these secondary metabolites. On the other hand, survival to adulthood was uniformly low across all populations, which could mean that plants from all five wild cabbage populations had sufficiently high levels of GS to negatively affect this herbivore. Although differences in plant quality mediated by primary and secondary metabolites may affect development of *D. radicum*, harsh conditions in the soil environment may generate enough variation to mask any potentially deleterious effects of plant quality on the performance of root flies. Previous work with *D. radicum* (Soler et al. 2007) on a related species, black mustard (*B. nigra*), with four root flies per plant, also reported low survival of flies to adulthood, whereas most studies with specialist aboveground feeding herbivores report high survival of the insects (e.g. Gols et al. 2008a,b, 2009). This suggests that the soil is a more hostile environment than the aboveground environment.

We did not find a difference in root biomass between the populations or between control and root fly damaged plants. This may be due to compensatory growth, a tolerance mechanism in which plants invest extra resources into root growth after root tissue damage (Karban and Baldwin 1997). Another possible explanation is that the number of root fly larvae per plant was not high enough to cause significant damage that would lead to a decreased root biomass. However, Soler et al (2007) did find an effect on root biomass, despite low survival of the root flies.

In summary, we have reported that five wild cabbage populations that grow along a linear transect of the English Channel coastline within 20 km of one other exhibit significant qualitative and quantitative differences in the expressions of GS, amino acids and sugars in root tissues. Given that the populations grow in close proximity, it is interesting to speculate about the factors that have generated and maintain these differences in chemical profiles of the roots and shoots. The plants may live up to 10 years in the wild, and, as we have previously reported, some of the populations (e.g. KIM, SAH) grow on high cliffs where they are continually exposed to prevailing westerly winds, whereas others (e.g. WIN) grow in more sheltered locations. Although the performance of a specialist root-feeding herbivore did not differ across the populations, we cannot exclude the possibility that the observed variation in chemistry has been shaped by differing selection pressures – including climate, and interactions with shoots and root antagonists such as herbivores and pathogens. Aboveground studies have shown that the abundance and identity of insects on aboveground plant tissues varies among the populations (Newton et al 2009).

A pilot study with three of the populations (WIN, KIM, OH) conducted in a garden plot at the NIOO showed that root flies primarily attack young plants with resulting differential mortality. We do not know what impact *D. radicum* attack has on wild cabbage plants in their natural habitat in Dorset, but it is likely that the insect will also affect the establishment of young plants. Future studies aim to quantify the rate of attack and density of root flies in the wild cabbage populations in England. Work underway will also show whether communities of belowground microbes and nematodes similarly differ on the five populations.

Few studies have examined chemical profiles of roots of different plant genotypes and linked the chemistry to the performance of a belowground herbivore. This study complements extensive previous work on the aboveground chemistry of wild cabbage, and adds to more comprehensive knowledge about wild cabbage populations and their interactions with associated insects.

Supplementary material

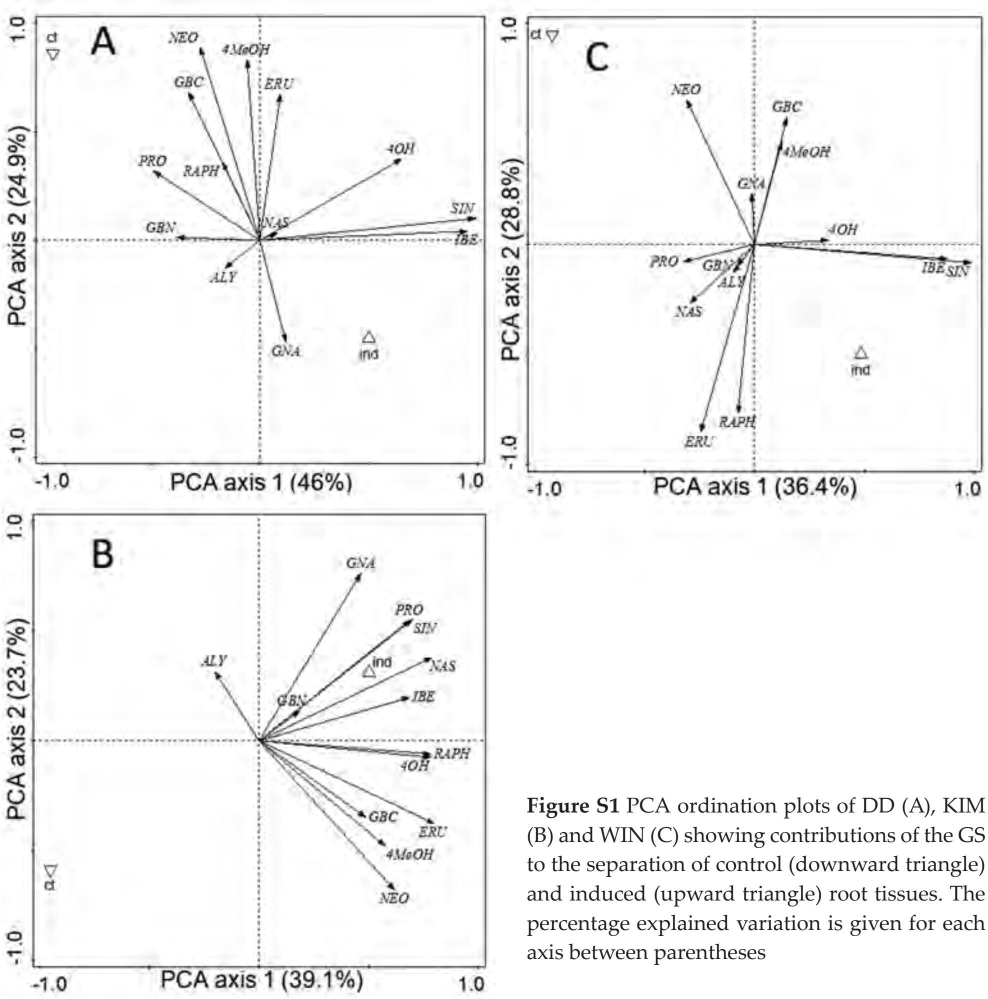


Figure S1 PCA ordination plots of DD (A), KIM (B) and WIN (C) showing contributions of the GS to the separation of control (downward triangle) and induced (upward triangle) root tissues. The percentage explained variation is given for each axis between parentheses

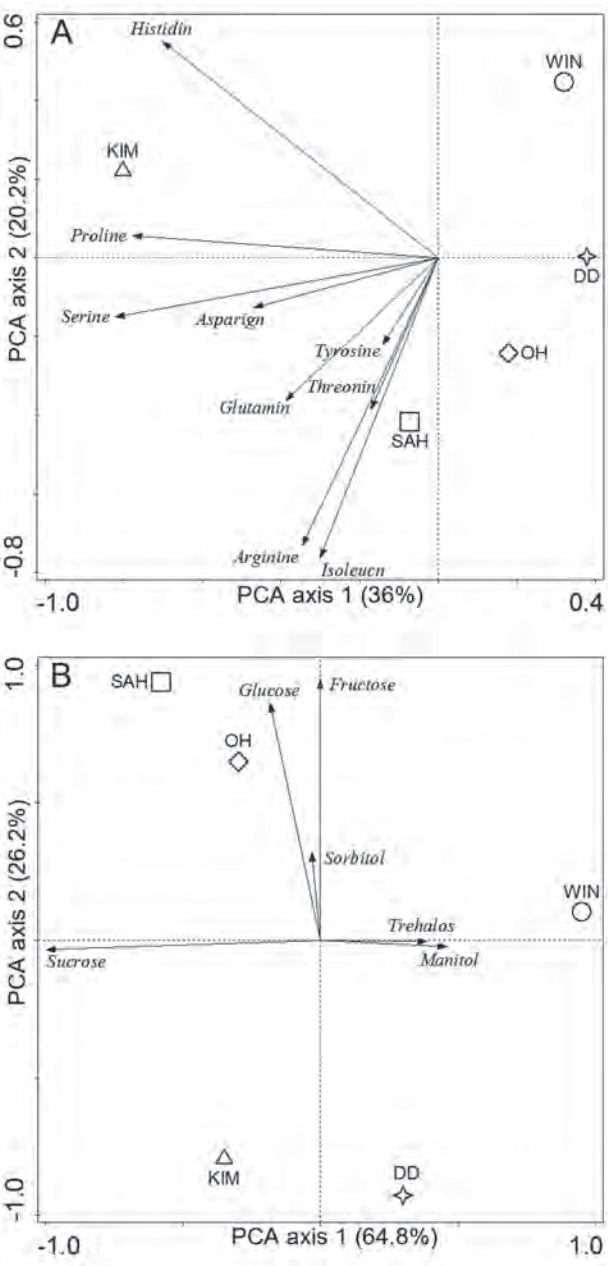


Figure S2 PCA ordination plot of the amino acids (A) and sugars (B) with the five wild cabbage populations projected as explanatory variables. The percentage explained variation is given for each axis between parentheses. DD: star, KIM: triangle, SAH: square, WIN: circle, OH: diamond

Chapter 5

Effects of population-related variation in primary and secondary metabolites on above- and belowground multi-trophic interactions

Moniek van Geem, Rieta Gols, Ciska E. Raaijmakers
and Jeffrey A. Harvey

Abstract

Insects feeding on aboveground and belowground tissues can influence each other through their shared plant. The outcome of aboveground-belowground interactions for the insects in terms of survival and development can be positive, negative or neutral, depending on changes in plant chemistry. Most studies have ignored genetic variation in plant chemistry within plant species, even though this affects the outcome of plant-insect interactions. Here we examined the effects of belowground herbivory on the performance of an aboveground herbivore and its endoparasitoid wasp, and if this was in turn linked to genotypic variation in the primary and secondary chemistry of the plants induced by the belowground herbivore. Insects were reared on three populations of wild cabbage (*Brassica oleracea*) plants that have been previously shown to exhibit both qualitative and quantitative differences in root and shoot defence chemistry. Plants were initially inoculated with larvae of a specialist belowground herbivore, the cabbage root fly *Delia radicum*. Two weeks later second instar larvae of a shoot-feeding specialist, the diamondback moth *Plutella xylostella*, were placed on the plants. In addition, cohorts of larvae were parasitized by *Cotesia vestalis*, a specialist solitary larval endoparasitoid of *P. xylostella*. Insect herbivore and parasitoid performance varied between the different wild cabbage populations, whether solely or in combination with sex and/or the type of herbivory. There was a correlation between insect performance and plant primary and secondary metabolites for male adult body mass of *D. radicum* and female and male development time of *P. xylostella*. There were also significant differences in primary and secondary metabolites between leaves and roots of the plants, both in terms of total concentrations and chemical profiles. No general patterns emerged on how plant population, aboveground herbivory and belowground herbivory influenced the glucosinolate, amino acid and sugar concentrations/profiles in the leaves and roots. Our results show that population-related variation in plant primary and secondary metabolites in plants adds an important extra layer of complexity to mechanistic studies of aboveground-belowground interactions.

Introduction

Plant-insect interactions have long underpinned ecological and evolutionary theory covering vastly different scales, from gene expression to the levels of communities and ecosystems (Hairston et al. 1960; Root 1973; Futuyma and Agrawal 2009). One of the major shortcomings of much of the research on plant-insect interactions is that until recently most studies focused primarily on the aboveground compartment. Over the past two decades, however, it has become increasingly recognized that a better understanding of aboveground processes needs to incorporate biotic interactions occurring in the rhizosphere (Masters and Brown 1992; van der Putten et al. 2001; Bezemer and van Dam 2005). Plant roots, which are essential for the uptake and storage of nutrients, also harbor many mutualists and/or antagonists such as nematodes, insects and pathogens (Waisel et al. 1996, Blossey and Hunt-Joshi 2003). Belowground organisms can play an important role in determining the composition of ecological communities, and, at greater spatial scales, larger ecosystem processes such as productivity and resilience (Wardle et al. 2004).

The outcome of interactions between organisms feeding on either above- or belowground plant tissues can be positive, negative or neutral, and has been the subject of many reviews (Masters et al. 1993; van der Putten et al. 2001; Blossey and Hunt-Joshi 2003; Bardgett and Wardle 2003; Wardle et al. 2004). Organisms in the aboveground (AG) and belowground (BG) compartments may indirectly interact with each other through changes in the quality and quantity of the shared plant that are mostly mediated by their feeding on plant tissues, evoking responses from the plant that are sometimes systemic (Bardgett et al. 1998; van der Putten et al. 2001; Soler et al. 2012). These biological interactions are to a large extent mediated by plant traits such as the production of phytochemicals (van Dam et al. 2003). Plants produce primary metabolites such as amino acids and carbohydrates that are essential for growth, development and reproduction (Schoonhoven et al. 2005). They also produce secondary metabolites that play no apparent role in fundamental physiological processes and have been shown to function as a defence against plant antagonists such as pathogens and herbivores (Hartmann 1996; Schoonhoven et al. 2005).

Primary and secondary metabolites are also important for consumers up the food chain because they influence plant quality for herbivores and even higher trophic levels up to the terminal end of the food chain (Scriber and Slansky 1981; Slansky 1986; Awmack and Leather 2002; Ode 2006). Primary metabolites in plants, for instance, provide nutrients that are essential for the development of the insects that feed on them. Nitrogen (N) is often a limiting nutrient for herbivores and thus concentrations of N may profoundly affect insect development (Awmack and Leather 2002; Fagan et al. 2002). Secondary metabolites are often repellent or even toxic to attacking herbivores (Scriber and Slansky 1981; Schoonhoven et al. 2005). However, secondary metabolites can also act as feeding or oviposition stimulants for well adapted specialist herbivores (Schoonhoven et al. 2005). In such cases, primary metabolites may be a more important determinant of plant quality for consumers up the food chain than secondary metabolites.

Concentrations of plant secondary metabolites can change in response to insect herbivory. Induction of secondary metabolites can occur at the attack site only (i.e. local response) or throughout the entire plant (i.e. systemic response), which can affect the performance of herbivores feeding on other plant organs in the same or opposite compartment (Masters et al. 2001; Bezemer and van Dam 2005). Herbivory can affect both primary chemistry (Gange and Brown 1989; Johnson et al. 2009) and secondary chemistry (Harvey et al. 2003, Bezemer et al. 2003, van Dam et al. 2005). Importantly, there is often considerable intraspecific genetic variation in the expression of secondary metabolites in plants (Hartmann 1996; Agrawal 2004; van Geem et al., 2013). Variation in the expression of plant secondary metabolites is presumably the result of a suite of differing biotic and abiotic selection pressures that may vary even at local scales. Genetic variation in plant chemistry is an important component of plant-insect interactions, but often overlooked in studies. In this experiment we looked at population-related differences in primary and secondary chemistry and whether these differences influence AG-BG interactions.

We examined the effect of herbivory by a BG specialist herbivore, *Delia radicum* L. (Diptera: Anthomyiidae), on primary and secondary chemistry in three different populations of wild cabbage, *Brassica oleracea* L. (Brassicaceae). In turn, we studied the effect of BG herbivory on the performance of an AG specialist herbivore, *Plutella xylostella* L. (Lepidoptera: Plutellidae), and its natural enemy, *Cotesia vestalis* H. (Hymenoptera: Braconidae). British populations of wild cabbage grow in Dorset and Devon and are known to differ profoundly in their chemical defence profiles (Mithen et al. 1995; Moyes et al. 2000; Gols et al. 2008b; Newton et al. 2009a; van Geem et al. 2013). As with other members of the Brassicales, cabbage plants produce secondary metabolites known as glucosinolates (hereafter GS; Halkier and Gershenzon 2006). The defence mechanism of plants producing GS entails the enzyme myrosinase, which is stored in separate cells. When cells are damaged, e.g. as a result of herbivore feeding, the GS and myrosinase come into contact with each other, resulting in the hydrolysis of GS into potentially toxic/deterrent breakdown products (Textor and Gershenzon 2009).

We hypothesized that 1) the performance of the AG trophic chain will be negatively affected by BG herbivory, 2) this negative effect will be correlated with changes in primary and secondary metabolites, and 3) primary and secondary chemistry will differ between AG and BG plant tissues (leaves and roots respectively), between the types of herbivory (AG or BG) and among the three wild cabbage populations. As far as we know, this is one of the first studies to compare the performance of AG and BG insects in different populations of a wild plant species and to link this with both primary and secondary metabolites.

Material and Methods

Plants

In this experiment we used three wild cabbage (*Brassica oleracea*) populations located in the UK, in the Dorset area near Swanage. Seeds were collected from multiple plant individuals per population, at Kimmeridge (KIM; 50^{0}35′N, 2^{0}03′W), Winspit (WIN; 50^{0}34′N, 2^{0}02′W) and Old Harry (OH; 50^{0}38′N, 1^{0}55′W). The seeds were germinated in germination soil (‘Lentse Stekgrond’, Lent, The Netherlands). When the seedlings were one week old, they were transplanted into 2-L pots (one plant per pot) filled with potting soil (‘Lentse Potgrond’ no. 4, Lent, the Netherlands).

The plants were left to grow for eight weeks in a glasshouse at 21±1 °C, 16L:8D, r.h. 70% in large trays (675 ×170 cm) that were automatically flooded for 20 min each day with water and nutrients (NH4 1.2, K 7.2, Ca 4.0, Mg 1.82, NO3 12.4, SO4 3.32, P 1.0, Fe 35.0, Mn 8.0, Zn 5.0, B 20.0, Cu 0.5, Mo 0.5 in mmol/L).

Insects

Delia radicum

The cabbage root fly (*D. radicum*) is a specialist root-feeding herbivore on members of the Brassicaceae. The larvae can weaken the root system and stem of a plant to such an extent that the plant may wilt and die. Adult females use GS to locate their host plants for oviposition (Roessingh et al. 1992).

The University of Rennes, France, provided root fly pupae to start a root fly culture at the NIOO in Wageningen. The French colony was started in September 2009 with root flies collected in the field (Le Rheu, Brittany, France, 48°07016″ N, 01°47041″ W). At the NIOO, cabbage root fly larvae were reared in a climate room (21±1 ^{0}C, 50-70% r.h., L16:D8) on a mixed diet of sugar, nutritional yeast and milk powder (1:1:1). Water was provided through wet filter paper. Adult root flies were offered fresh pieces of turnip/rutabaga on moist filter paper in a Petri dish for females to oviposit on. After one day, the Petri dish was removed and the eggs were collected by rinsing the pieces of turnip/rutabaga with water and collecting the eggs in a fine sieve. The eggs were then spread on top of intact turnips which were placed in plastic containers filled with coarse sand in which the larvae can pupate after eating their way through the turnip in about 3 weeks.

Plutella xylostella

Larvae of the diamondback moth *P. xylostella* are, like *D. radicum*, specialist feeders on plant species in the Brassicales, with GS acting as feeding and oviposition stimulants (Talekar and Shelton 1993). In large numbers the larvae can cause serious damage to plants by defoliation of the shoots.

P. xylostella larvae used in the experiments were obtained by offering cabbage plants for oviposition to adults in the culture maintained at the Laboratory of Entomology, Wageningen University. The eggs were left on the leaves to hatch. Second instar larvae were transferred to predetermined experimental plants (see below).

Cotesia vestalis

C. vestalis is a solitary koinobiont endoparasitoid, i.e. the female wasp lays a single egg into the body of the host caterpillar which continues feeding and growing during parasitism. This parasitoid is only known to attack larvae of *P. xylostella*. When it is fully grown, the mature wasp larva chews its way out of the dying host caterpillar and spins a cocoon. Female wasps used for parasitism originated from a *C. vestalis* culture maintained on *P. xylostella* feeding on cultivated cabbage (*B. oleracea* var. gemmiferea, cv. Cyrus) at the Laboratory of Entomology, Wageningen University.

Experimental design

Plants of each of the three populations were assigned to one of six treatments: 1) no herbivores, 2) only root flies, 3) only *P. xylostella*, 4) only parasitized *P. xylostella*, 5) root flies and *P. xylostella*, 6) root flies and parasitized *P. xylostella.* There were ten plants per treatment per population and a total of 180 plants. Each individual plant was covered with a sleeve net (BugDorm, Megaview Science, Taiwan) of 100x66 cm (LxW) to confine the insects to their respective plants.

Plants from treatments with root flies (treatments 2, 5 and 6) were each inoculated with 8 newly-hatched L1 root fly larvae. Sixteen and seventeen days after inoculation with root fly larvae, non-parasitized *P. xylostella* and parasitized larvae (15 L2 larvae per plant), respectively, were placed on the assigned plants. Second instar *P. xylostella* larvae were individually parasitized by *C. vestalis* females by offering hosts to wasps in a vial until insertion of the ovipositor was observed. Insects were allowed to move and feed freely on their host plants until they had completed their larval development.

Pupae from *P. xylostella* were collected one day after they had pupated and placed individually in labelled glass vials. As soon as an adult emerged, the date and time of eclosion and its sex were recorded and the vial was placed in a freezer to kill the adult. Adults were dried in an oven at 70 ^{0}C for three days to determine their dry body mass.

Cocoons of *C. vestalis* were collected and processed similarly as described for the adult moths One day after most of the moths or wasps had pupated, leaves of the plants were sampled for chemical analysis (see below).

Emerged adult root flies were counted to determine their survival. In addition, sex, body weight and development time were recorded. Following root fly eclosion, the roots were sampled for chemical analysis.

Chemical analyses

Six leaves that had experienced herbivory were randomly sampled per plant. Using a cork borer leaf discs (Ø 1 cm) were excised from the leaves. From young, small leaves 1 disc was sampled and from older, larger leaves 2 discs. For each individual plant the discs were pooled, wrapped in tin foil, labeled and immediately frozen in liquid nitrogen. All the samples were freeze-dried and pulverized with a grinding machine (Retch, type MM 301). The pulverized material was then weighed into 2mL Eppendorf tubes (50 ± 2mg).

To collect root material, roots of all plants were removed from the soil. After rinsing with water to remove soil, the roots were placed in paper bags and stored at -20 °C until further processing. The roots were freeze dried for 4 days and then cut into small pieces of which a representative sample (parts from both thick and thin roots) was collected for chemical analysis. Those samples were ground to powder with the grinding machine.

For quantification of GS, amino acids and soluble sugars, one global 70% methanol extraction was conducted (see van Geem et al. 2015).

Glucosinolate analysis

GS were desulfatazed with sulfatase (Sigma type H-1 from Helix pomata) on a DEAE-Sephadex A25 column, and separated by high-performance liquid chromatography (HPLC) with a acetonitrile-water gradient on a reversed phase Alltima C18 column (150 x 4.6 mm). The GS were detected with UV diode array at a wavelength of 229 nm. Sinigrin in 5 different concentrations (63 μM – 625 μM) was used as an external standard for the quantification of the GS. Individual GS were identified based on their retention times and UV spectra compared to those of the standards (EC Community Bureau of Reference, Brussels, Belgium, BCR-367R). Final concentration (nMoles/mg) were calculated by correcting for the volume and dry mass of the extract and original tissue. For a more detailed description see van Geem et al (2015).

Soluble sugar analysis

Sugars were separated using ion-exchange HPLC with a CarboPac PA1 main column (2 x 250 mm) and a CarboPac PA1 guard column (2 x 50 mm). The standard curves of 11 sugars (2.5-10 ppm) were used as reference.

Amino acid analysis

Amin acids were separated using ion-exchange HPLC with an AminoPac PA10 main column (2 x 250 mm) and an AminoPac PA10 guard column (2 x 50 mm) The standard curves of 20 amino acids (1-8 µM) were used as reference.

Statistical analyses

Insect performance variables were analyzed using SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). Survival data were analyzed with binary logistic regression. Development time and adult body mass were analyzed using a mixed model approach with population, treatment and sex as fixed factors and cage ID as a random factor to account for the fact that the data obtained from insects developing within the same cage were not independent. Estimation of the model terms was based on Restricted Maximum Likelihood (REML).

We tested for correlations between insect performance and plant chemistry, both for the performance data when a herbivore was alone (A or B) and when there was another herbivore in the opposite compartment (A+B). This was done for all three performance variables (survival, development time, adult body mass). For development time and adult body mass separate analyses were performed for female and male insects. Outliers in the adult body mass data for *D. radicum* that fell outside the range of mean ± 3 x standard error were removed prior to analysis.

Both quantitative (total concentrations) and qualitative (chemical profiles) aspects of the primary and secondary chemistry were analyzed. Total levels of the primary and secondary metabolites were analyzed with univariate analysis using SPSS version 22 (IBM SPSS Statistics). Two-way ANOVA was used with plant population, AG herbivory, and BG herbivory as fixed factors. Where needed, the data were log- or square-root-transformed to meet assumptions of normality and homogeneity of variance. There are three classes of glucosinolates (GS), which differ in the origin of the amino acid from which they have been derived (Halkier and Gershenzon 2006): indole, aromatic and aliphatic GS. In many leaf samples the aromatic GS nasturtiin could not be detected and, therefore, this compound was not included in the analysis of the total concentrations of the different GS classes in the leaves.

Both the chemical profiles of the plant tissues and insect-chemistry correlations were analyzed with multivariate analysis using redundancy analysis (RDA) with Canoco 5 (5.03, ter Braak and Smilauer). The chemical profiles of the leaves and roots were analyzed for differences between the plant populations (KIM, WIN, OH) and herbivory treatments (C = control, A = only aboveground herbivory present, B = only belowground herbivore present, A+B = both above- and belowground herbivore present). All chemical data were log-transformed and mean centered.

Results

AG insect performance

Plutella xylostella

Survival of the AG herbivore *P. xylostella* was unaffected by type of herbivory (χ^2_1=0.31, P=0.58), plant population (χ^2_2=1.67, P=0.43), and the interaction between these two factors (χ^2_2=0.4, P=0.82) (Fig. 1A). BG herbivory did not affect development time ($F_{1,53.8}$=0.08, P=0.78) or adult body mass ($F_{1,56}$=1.02, P=0.32) of *P. xylostella*, but plant population did. Development time was fastest on WIN plants and slowest on KIM plants ($F_{2,53.8}$=5.66, P=0.006), for females and males equally ($F_{1,540}$=0.67, P=0.41) (Fig. 1B). Adult body mass was higher for females than males ($F_{1,575}$=1271.8, $P \leq 0.001$), and the diamondback moths had highest adult body mass on KIM, followed by OH and then WIN plants (($F_{2,54.9}$=3.28, P=0.045; Fig. 1C).

Cotesia vestalis

Survival of the parasitic wasp *C. vestalis* was significantly affected by an interaction between plant population and type of herbivory (plant population: χ^2_2=1.58, P=0.45; type of herbivory: χ^2_1=0.5, P=0.48; interaction: χ^2_2=7.64, P=0.02). Parasitoid survival on WIN was unaffected by BG herbivory, whereas for KIM BG herbivory was associated with higher survival. Conversely, on OH survival was lower on plants with BG herbivory (Fig. 2A). Sex, plant population, type of herbivory and an interaction between plant population and type of herbivory also significantly affected the development time of the wasps (sex: $F_{1,436}$=12.2, $P \leq 0.001$; plant population: $F_{2,437}$=6.3, P=0.002; type of herbivory: $F_{1,437}$=12.15, $P \leq 0.001$; interaction plant population x type of herbivory: $F_{2,437}$=6.04, P=0.003). Males developed faster than females. For KIM and OH, wasps developed faster in the presence of the BG herbivore, whereas for WIN the wasps developed faster in the absence of the BG herbivore (Fig. 2B). The adult body mass of females was higher than males ($F_{1,444}$=101.09, $P \leq 0.001$). Neither plant population ($F_{2,444}$=0.32, P=0.73) nor BG herbivory ($F_{1,444}$=0.38, P=0.54) affected adult body mass (Fig. 2C).

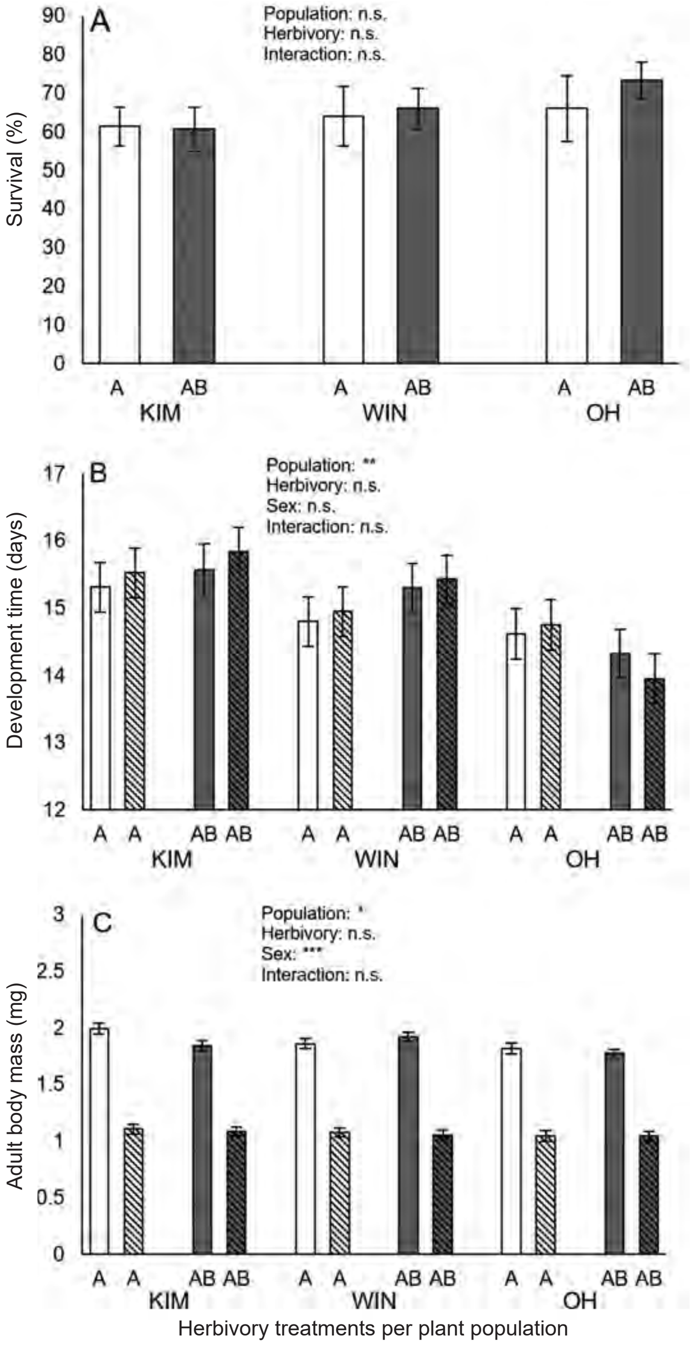


Figure 1 Mean (± SE) survival (A), development time (B) and adult body mass (C) of *Plutella xylostella* on wild cabbage plants origination from three populations (KIM, WIN and OH) that were exposed to the diamondback moth alone (white bars, A group on x-axis) or were also exposed to the BG herbivore *D. radicum* (grey bars, AB group x-axis. Open bars represent the data for the females and dashed bars the data for the males. Note that the y-axis for development time (B) starts at 12.

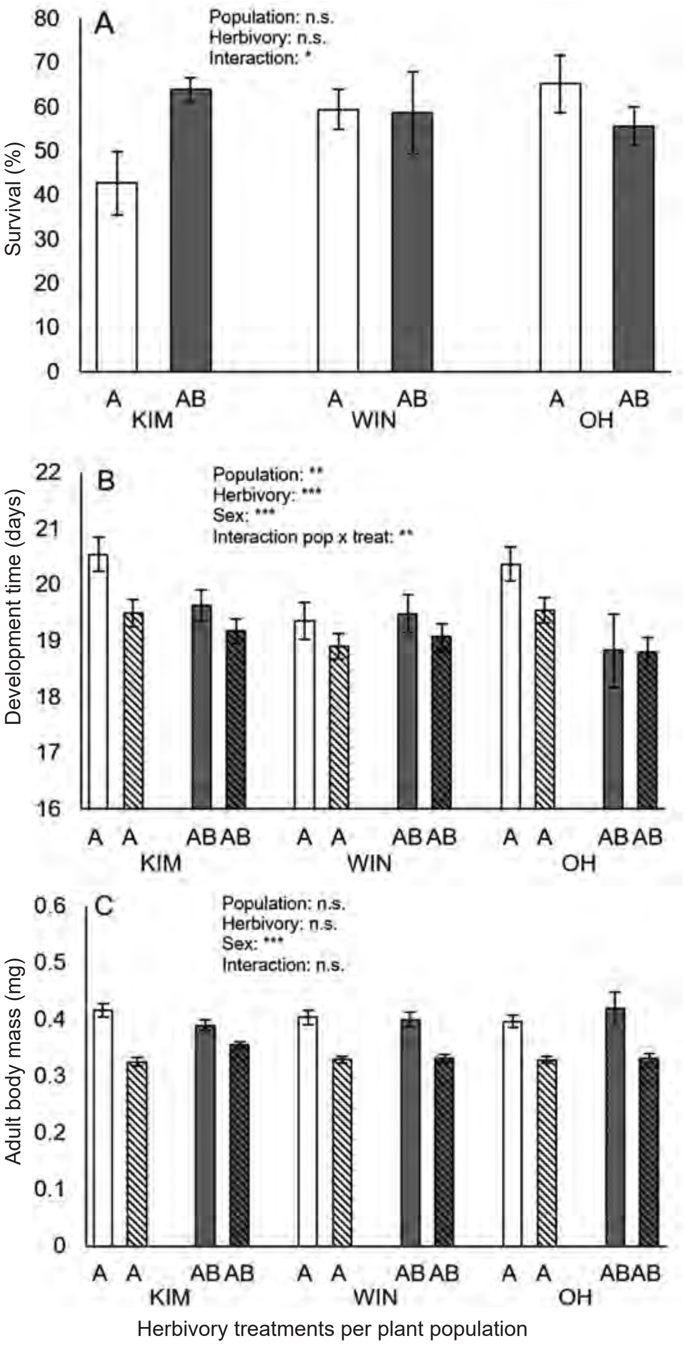


Figure 2 Mean (± SE) survival (A), development time (B) and adult body mass (C) of *Cotesia vestalis* on wild cabbage plants origination from three populations (KIM, WIN and OH) that were exposed to the parasitized *P. xylostella* alone (white bars, A group on x-axis) or were also exposed to the BG herbivore *D. radicum* (grey bars, AB group x-axis. Open bars represent the data for the females and dashed bars the data for the males. Note that the y-axis for development time (B) starts at 16.

Delia radicum

The performance of the belowground herbivore in response to AG herbivory and the different wild cabbage populations was also recorded. Survival of the root flies was lowest on WIN and highest on OH plants (χ^2_2=6.67, *P*=0.036; Fig. 3A). Neither herbivory treatment (χ^2_1=2, *P*=0.16) nor the interaction between plant population and herbivory treatment affected survival of the root flies (χ^2_2=0.66, *P*=0.72). Development time of the root flies was significantly affected by the type of herbivory and sex but also by the three-way interaction between plant population, type of herbivory and sex (plant population: $F_{2,54.7}$=2.63, *P*=0.08; type of herbivory: $F_{1,54.7}$=7.98, *P*=0.007; sex: $F_{1,390}$=60.6, *P*≤0.001; interaction: $F_{2,390}$=4.43, *P*=0.013). In general, males developed faster than females. For KIM and WIN plants, root flies developed faster in the absence of the AG herbivore, but this was not apparent when insects were reared on OH plants (Fig. 3B). Adult body mass of *D. radicum* varied significantly with sex ($F_{1,275}$=153.94, *P*≤0.001) and plant population ($F_{2,45.2}$=5.5, *P*=0.007) but was not affected by the presence of the AG herbivore ($F_{1,47.8}$=0.08, *P*=0.78). Female root flies were larger than males. Root flies had the highest body mass when they developed on KIM, followed by OH and WIN (Fig. 3C).

Insect performance and chemistry

For the AG herbivore *P. xylostella* we found significant correlations between female and male development time and chemistry, but only in the presence of the BG herbivore (female A+B: *F*=3.3, *P*=0.029; female A: *F*=0.9, *P*=0.418; male A+B: *F*=4, *P*=0.006; male A: *F*=2, *P*=0.098). For females the development time was positively correlated with a.o. the GS progoitrin, the amino acid tyrosine and the sugar trehalose, while being negatively correlated with a.o. the GS gluconapin, the amino acid histidine and the sugar sorbitol (Fig. S1A). For males the development time was positively correlated with a.o. the GS glucoiberin, the amino acid glutamate and the sugar glucose, while being negatively correlated with a.o. the GS gluconapin, the amino acid histidine and the sugar sorbitol (Fig. S1B). Here, positive correlations mean that the development time is longer when concentrations of these compounds increase, whereas negative correlations mean that the development time is longer when concentrations of these compounds decrease. In other words, negative correlations are associated with a faster development. We did not find any correlations between insect performance and plant chemistry for the parasitoid *C. vestalis*.

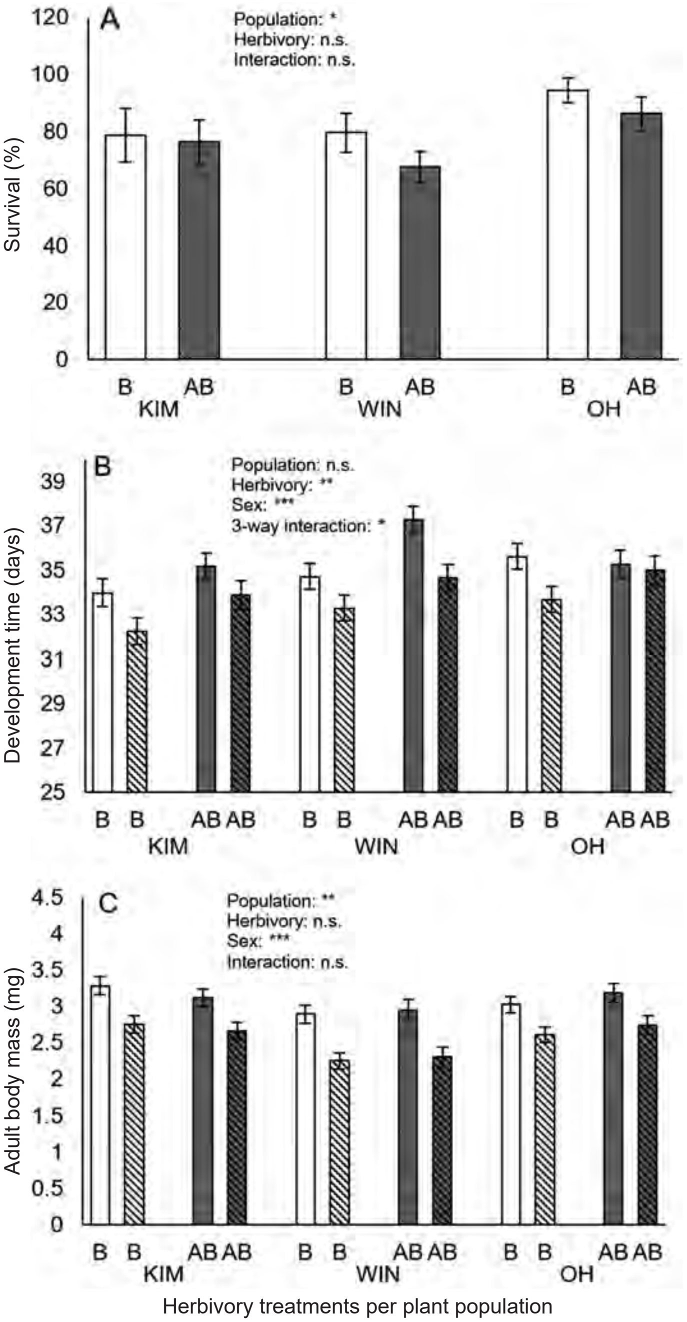


Figure 3 Mean (± SE) survival (A), development time (B) and adult biomass (C) of *Delia radicum* on wild cabbage plants originating from three populations (KIM, WIN and OH) that were exposed to the root fly alone (white bars, B group on x-axis) or were also exposed to the AG herbivore *P. xylostella* (grey bars, AB group on x-axis). Open bars represent the data for the females and dashed bars the data for the males. Note that the y-axis for development time (B) starts at 25.

For the BG herbivore *D. radicum* we found a correlation between male adult body mass and plant chemistry, but only when the AG herbivore was absent (A+B: F=2, P=0.078; B: F=2.7, P=0.007). Male adult body mass was negatively correlated with the majority of the individual compounds of GS, amino acid and sugar, but also positively correlated with the GS gluconasturtiin, the amino acids glutamate, isoleucine and tryptophan, and the sugar sucrose (Fig. S1C).

Plant chemistry

Glucosinolates

In total fourteen GS were detected, of which three were only present in the root tissues and not in the leaf tissues (gluconapoleiferin, glucoerucin and an unknown sulfinyl). Total GS concentrations differed between the leaves and the roots (ANOVA: $F_{1,178}$=92.8, P≤0.001) with higher concentrations in the root tissues than in the leaf tissues. The glucosinolate profiles of leaves and roots were also significantly different from each other (RDA: F=25.5, P=0.001). Concentrations of glucobrassicin and gluconapin were higher in the leaves, whereas concentrations of other GS, especially gluconasturtiin, were higher in the roots. Figure S2 shows the GS profiles of the leaves (A) and roots (B) from the three wild cabbage populations.

LEAVES

The total GS concentration in the leaves differed significantly between the three plant populations ($F_{2,48}$=60.93, P≤0.001) with the highest total concentrations found in WIN, followed by OH and then KIM (Fig. 4A). Total GS concentration was neither affected by AG herbivory ($F_{1,48}$=0.79, P=0.38) nor by BG herbivory ($F_{1,48}$=0.25, P=0.62). For the total indole GS concentration we found significant effects of AG herbivory ($F_{1,48}$=20.43, P≤0.001), BG herbivory ($F_{1,48}$=7.33, P=0.009) and plant population ($F_{2,48}$=6.23, P=0.004) but no significant interaction effects. In all three populations, indole concentrations were highest when both the AG and BG herbivore were present. For the total aliphatic GS concentration there were significant effects of AG herbivory ($F_{1,48}$=10.12, P=0.003), plant population ($F_{2,48}$=77.3, P≤0.001) and the interaction between those two factors (($F_{2,48}$=3.88, P=0.027). Generally, AG herbivory decreased concentrations of aliphatic GS, but the relative effect of AG herbivory was not the same in each of the populations. Aliphatic GS concentrations were low in KIM, intermediate in OH, and high in WIN plants. Belowground herbivory did not have an effect on aliphatic GS concentration in the leaves ($F_{1,48}$=2.96, P=0.092).

The GS profiles in the leaf tissues was significantly affected by plant populations and the interactions between herbivory treatment and plant population (plant population: F=28.3, P=0.001; herbivory treatment: F=1.4, P=0.165; interaction: F=2.0, *P*=0.003), indicating that the effect of the type of herbivory differed between the three wild cabbage populations. Within populations, the GS profiles of the four herbivory treatments significantly differed from each other for KIM (*F*=3.2, *P*=0.005) and OH (*F*=2.7, *P*=0.003), but not for WIN (*F*=1.8, *P*=0.06).

ROOTS

There were significant effects of AG herbivory ($F_{1,48}$=4.61, *P*=0.037), BG herbivory ($F_{1,48}$=18.67, *P*≤0.001), plant population ($F_{2,48}$=11.73, *P*≤0.001) and an interaction between these three factors ($F_{2,48}$=7.5, *P*=0.001) on the total GS concentration in the root tissues (Fig. 4B). There was a similar pattern for KIM and OH plants where total GS concentrations were highest for control plants, lowest in plants that were exposed to root fly feeding either alone or together with AG caterpillar feeding, and intermediate for plants exposed to only AG feeding. For WIN, compared with the control treatment, total GS concentrations were higher in plants exposed to either AG or BG herbivory, whereas they were lower in plants exposed to both AG and BG herbivory. Similar to the total GS concentration, the total aliphatic GS concentration was significantly affected by an interaction between plant population, AG herbivory and BG herbivory (AG herbivory: $F_{1,48}$=3.38, *P*=0.072; BG herbivory: $F_{1,48}$=22.55, *P*≤0.001; plant population: $F_{2,48}$=31.37, *P*≤0.001; interaction: $F_{2,48}$=5.55, *P*=0.007). Highest total aliphatic concentrations were found in plants from WIN that had been subjected to AG herbivory or BG herbivory; lowest total aliphatic GS concentrations were found in plants from KIM that had been subjected to BG herbivory alone. The total concentrations of indole and aromatic GS were only significantly affected by BG herbivory (indole: $F_{1,48}$=4.68, *P*=0.035; aromatic: $F_{1,48}$=16.56, *P*≤0.001). For indole GS, higher concentrations were found in the presence of the BG herbivore, whereas for aromatic GS BG herbivory seemed to reduce their concentrations. The GS profiles in the root tissues were significantly affected by plant population (*F*=11.1, *P*=0.001), but not by herbivory treatment (*F*=1.5, *P*=0.094) or their interaction (*F*=1.4, *P*=0.116).

Amino acids

We detected fifteen amino acids in total, two of which (lysine and alanine) were only found in leaf tissues, whereas cystine and tryptophan were only found in root tissues in relatively low concentrations. Total amino acid concentrations differed between the leaves and roots ($F_{1,178}$=31.89, *P*≤0.001), with higher concentrations in the leaves. The amino acid profiles of the leaves and roots were also significantly different from each other (*F*=31.1, *P*=0.001). Leaves had higher concentrations of serine, lysine, proline, arginine, alanine, threonine and glutamate, whereas the roots had higher concentrations of histidine, glutamine, asparagine, cystine, tyrosine, isoleucine, tryptophan and aspartate. Figure S2 shows the amino acid profiles of the leaves (C) and roots (D) from the three wild cabbage populations.

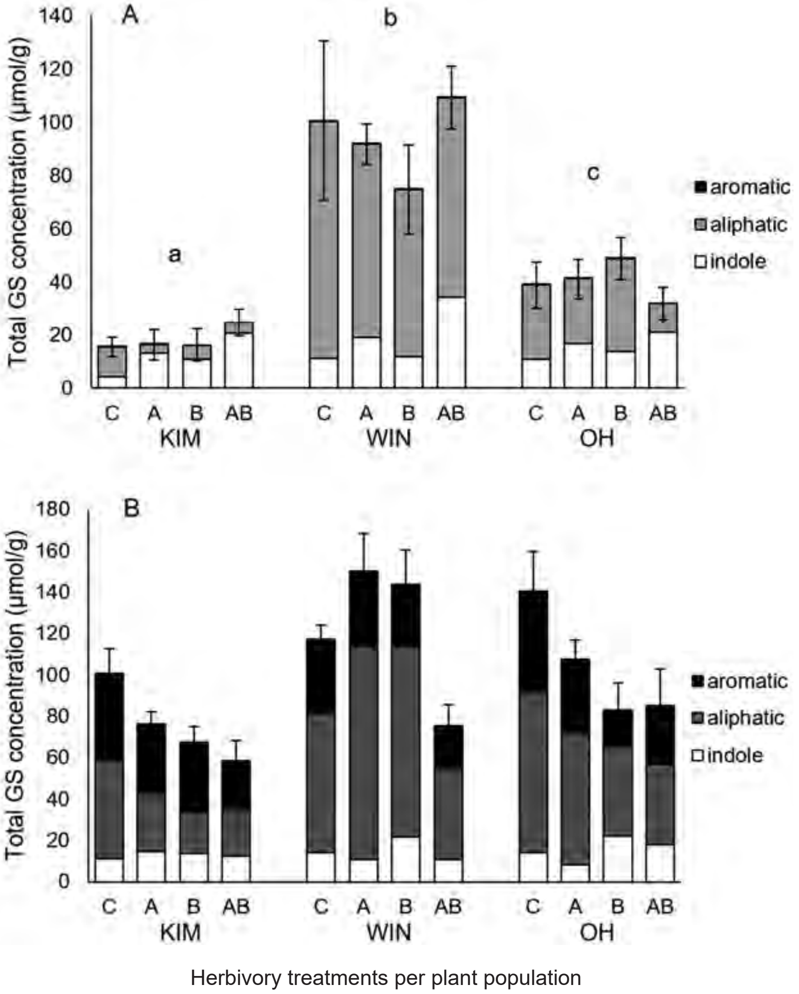


Figure 4 Total glucosinolate concentrations (mean ± SE) in the leaves (A) and roots (B) in wild cabbage plants originating from three populations (KIM, WIN and OH). On the x-axis are the four herbivory treatments: control (C), only aboveground herbivore (A), only belowground herbivore (B), and above- and belowground herbivore (AB). The colour shades in the graphs refer to the three different GS classes: white = indole, grey = aliphatic, black = aromatic. Different letters indicate significant differences between populations

LEAVES

Total amino acid concentrations in the leaves (Fig. 5A) were negatively influenced by the presence of the AG herbivore ($F_{1,48}$=31.4, $P \leq 0.001$). Plant population and BG herbivory did not have an effect on the total amino acid concentration (plant population: $F_{2,48}$=2.87, P=0.067; BG herbivory: $F_{1,48}$=1.16, P=0.287). The amino acid profiles of the leaf tissues were significantly affected by herbivory treatment, plant population and their interactions (herbivory treatment: F=2.8, P=0.001; plant population: F=5.8, P=0.001; interaction: F=2.3, P=0.001).

ROOTS

Total amino acid concentration in the roots (Fig. 5B) was significantly affected by BG herbivory, plant population and an interaction between plant population, BG herbivory and AG herbivory, which makes it difficult to interpret the data, showing that there are no consistent patterns between the populations in their responses to the three treatments (AG herbivory: $F_{1,48}$=0.0, P=0.995; BG herbivory: $F_{1,48}$=4.92, P=0.031; plant population: $F_{2,48}$=4.04, P=0.024; three-way interaction: $F_{2,48}$=5.24, P=0.009). The amino acid profiles of the root tissues were significantly affected by herbivory treatment, plant population and their interaction (herbivory treatment: F=2.4, P=0.001; plant population: F=2.4, P=0.008; interaction: F=1.6, P=0.014).

Sugars

Five sugars were present in both the leaves and the roots, although the leaf concentrations were considerably higher than the root concentrations. Total sugar concentrations differed between the leaves and the roots ($F_{1,178}$=296.5, $P \leq 0.001$), with higher concentrations in the leaves. The sugar profiles of the leaves and roots were also significantly different from each other (F=169, P=0.001). Leaves were characterized by higher concentrations of glucose, sorbitol and fructose, whereas roots had higher concentrations of trehalose and sucrose. Figure S2 shows the sugar profiles of the leaves (E) and roots (F) from the three wild cabbage populations.

LEAVES

Total sugar concentrations in the leaves (Fig. 5C) were higher in the absence of the AG herbivore ($F_{1,48}$=24.14, $P \leq 0.001$), but unaffected by BG herbivory ($F_{1,48}$=1.87, P=0.178) or plant population ($F_{2,48}$=3.1, P=0.054). The sugar profiles of the leaf tissues were significantly affected by herbivory treatment (F=4.7, P=0.001) but not by plant population (F=2.3, P=0.071) or the interaction between these two factors (F=1.1, P=0.383).

ROOTS

Total sugar concentrations in the roots (Fig. 5D) were higher in the presence of the BG herbivore ($F_{1,48}$=5.59, P=0.022), but not affected by AG herbivory ($F_{1,48}$=0.03, P=0.862) or plant population ($F_{2,48}$=0.443, P=0.645). The sugar profiles of the root tissues were significantly affected by herbivory treatment (F=3.3, P=0.002) but not by plant population (F=1.2, P=0.307) or the interaction between these two factors (F=1.2, P=0.247).

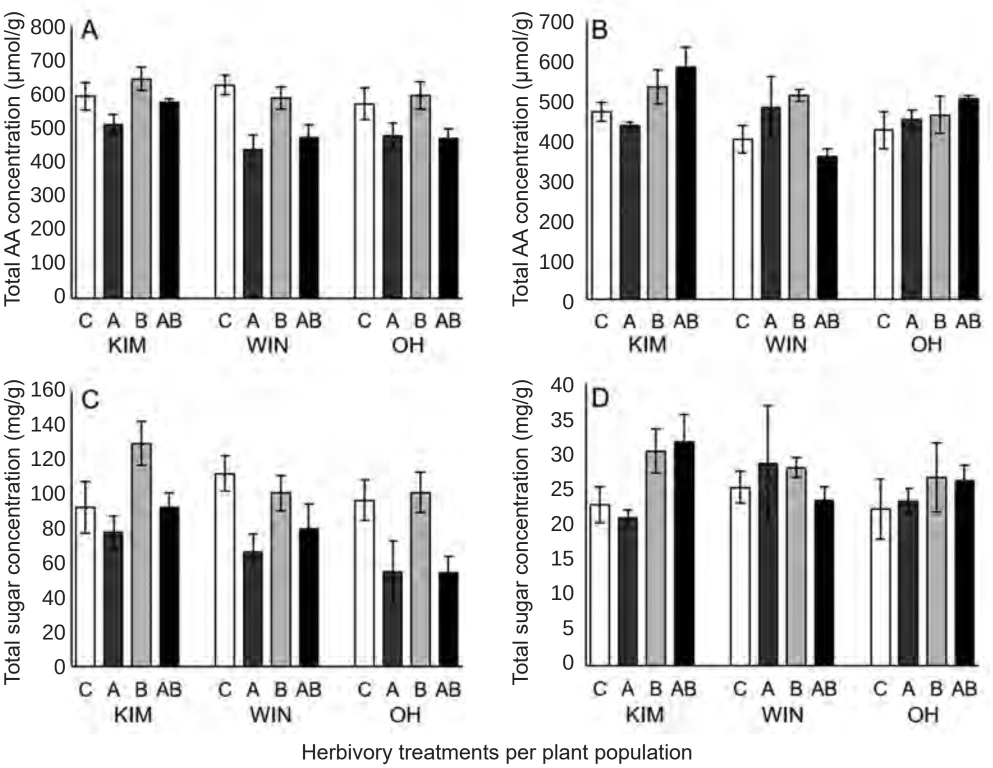


Figure 5 Mean (± SE) total concentrations of primary metabolites in the leaves and roots. A & B: total amino acids in the leaves and roots respectively; C & D: total sugars in the leaves and roots respectively. On the x-axis are the four herbivory treatments: control (C, white bars), only aboveground herbivore (A, dark grey bars), only belowground herbivore (B, light grey bars), and above- and belowground herbivore (AB, black bars)

Discussion

Insect performance

The results show that the specialist insects used in this study are quite well adapted to qualitative changes in the plant mediated by the presence or absence of insects in the opposite compartment. That being said, BG herbivory by *D. radicum* exhibited non-linear effects up the food chain, effectively bypassing the AG herbivore, *P. xylostella*, but affecting its endoparasitoid, *C. vestalis*, in terms of survival and development time. The effect of BG herbivory on survival and development time of *C. vestalis* was variable. Parasitoid survival depended on the population on which its host had been feeding, with neutral, negative and positive effects on WIN, OH and KIM plants respectively; parasitoids also developed faster in the presence of *D.*

radicum on KIM and OH plants but not on WIN plants. Belowground herbivory on KIM plants thus had a positive effect on the performance of *C. vestalis*, but there were no other patterns visible. These results clearly illustrate the complex nature of plant population and AG and BG herbivore-related effects on the performance of higher trophic levels.

For the BG herbivore *D. radicum*, development time was significantly slower in the presence of the AG herbivore *P. xylostella* even though *P. xylostella* larvae were only transferred to the plants in the final larval developmental stage of *D. radicum*. This result reveals that the final stage of larval development of the root fly is highly susceptible to changes in host plant quality that may be mediated by the AG herbivore. The larvae of most holometabolous insect herbivores consume >80% of their food in the final instar and are therefore very prone to changes in plant quality during this feeding stage. Indole GS have been found in previous studies to most deleteriously affect the development of specialist herbivores (Gols et al. 2008b; Abdalsamee and Muller 2012). However, concentrations of indole GS in the roots were more affected by BG herbivory than by AG herbivory. Therefore, understanding how AG herbivory may have affected the performance of *D. radicum* remains elusive. It may be that the effects of AG and BG herbivory are cumulative and when combined that they exceed some critical threshold beyond which insect performance is affected.

Development time and adult mass of the AG herbivore differed among the three populations of wild cabbage, and survival of its parasitoid as well as development time also differed with cabbage population. Moreover, fitness-related traits, most notably survival, of the root fly also differed among the different cabbage populations. Similar results on wild cabbages have been shown in several other studies with other species of specialist (Harvey et al. 2007) and generalist (Gols et al. 2008b) AG and/or BG herbivores. Harvey and Gols (2011b) found that development of a generalist herbivore, the cabbage moth *Mamestra brassicae*, was more negatively affected when developing on WIN plants than was development of its parasitoid, *Microplitis mediator*, a pattern which is opposite to what we found here with *P. xylostella* and *C. vestalis* (where the parasitoid was more negatively affected by BG herbivory than its host).

For the specialist insects studied here, genetic variation in the quality of the plant populations is probably much more important than the more subtle effects within individual plants that are mediated by herbivory in the opposite compartment. This further suggests that differences in plant quality at the population level are much larger than differences within the individual plants, whether the plants are attacked by BG herbivores or not. Previous studies with BG herbivores and wild Brassicaceous plants (e.g. Soler et al. 2005; 2007b) compared AG insect performance and behaviour on black mustard (*Brassica nigra*) plants in the presence or absence of *D. radicum* and found significant effects up to the fourth trophic level (Soler et al. 2005). However, those studies obtained mustard plants from a single Dutch population. Had they examined BG herbivory across different plant populations, they may also have found that plant population-related effects are more apparent on AG insect performance than BG herbivory. It is also important to note that *P. brassicae* is a much larger macro-invertebrate herbivore than *P. xylostella* and that it removes much more tissue from the host plant; how this affects BG induction is open to question.

Primary and secondary metabolites

Primary and secondary chemistry in *B. oleracea* differed between leaves and roots, between the wild cabbage populations and, to a lesser extent, between the different herbivory treatments. Total GS concentrations were higher in root than in the leaf tissues, whereas this pattern was reversed for the amino acids and sugars. The chemical profiles of the leaves and roots were also different in terms of the presence and relative concentrations of individual compounds. Although previous work on the same wild cabbage populations found that concentrations of GS were higher in the leaves than in the roots (van Geem et al. 2013), other studies have found that in general GS occur in higher concentrations in the roots than in the leaves (Kaplan et al. 2008; van Dam et al. 2009). Once again, this reveals the complex nature of plant physiology and effects of insect herbivores. How this plays out in the field under natural conditions where abiotic parameters vary continually and in different directions is an area that is in urgent need of investigation.

In the leaves, the total concentrations of GS differed between the three wild cabbage populations and were not affected by herbivory, whereas in the roots total GS concentrations differed between the populations and also in response to the herbivore treatments. Differences in GS profiles of AG and BG plant tissues among the populations has already been shown (Harvey et al. 2007; Gols et al. 2008a; Gols et al. 2008b; Harvey and Gols 2011b; Harvey et al. 2011; van Geem et al. 2015). In the KIM and OH population total GS concentrations in the roots tended to be reduced when plants were exposed to AG or BG herbivore treatments or both, whereas in WIN the response to herbivory was more idiosyncratic. Considering the GS profiles, in the leaves they differed between the populations and also interacted with herbivore treatment, whereas in the roots they only differed between the populations. This is opposite for what we found for the total GS concentrations.

Total GS concentrations in the leaves were highest in WIN and lowest in KIM, a finding that has been reported in previous studies (Gols et al. 2008b; Harvey and Gols 2011b; van Geem et al. 2013). Similarly, total GS concentrations in the roots were highest in WIN and OH, and lower in KIM. These differences may reflect differing susceptibility of the wild cabbage populations to attack from AG antagonists including herbivores and pathogens. British wild cabbage plants are interesting in that they only grow in chalky soils in generally rugged coastal habitats; some of these locations are very exposed to prevailing winds whereas others are not. In turn, the exposure of the plants to prevailing winds will certainly affect the ability of herbivores to find and exploit these plants. Of the Dorset populations studied, WIN plants grow in the most secluded location and large populations of herbivores (e.g. larvae of pierid butterflies, *Evergestis* spp, whiteflies) have been occasionally found on them. On the other hand KIM plants grow along a very exposed eroding cliff face and this location is almost continually buffeted by strong winds along the English Channel; consequently very few insects have been found on these plants (J. Harvey and R. Gols, unpublished observations).

Several studies have examined the reciprocal effects of AG and BG herbivory on primary and secondary chemistry in plants. For example, Kaplan and colleagues (2008) found that in tobacco plants AG herbivory only affected leaf chemistry, whereas BG herbivory affected both leaf and root chemistry. Their findings were supported by a meta-analysis on induced defences in a wide range of plant-herbivore systems which showed that generally leaf herbivory induces leaf tissue, whereas root herbivory induces both leaf and root tissue (Kaplan et al. 2008). However, we did not find this pattern in our study with wild cabbage, showing that there may be considerable variation among different plant-herbivore associations and that it is difficult to generalize the effects of AG and BG herbivory on induction of plant secondary compounds.

In terms of primary metabolites, amino acid concentrations were higher in the leaves than in the roots. In the leaves, the total concentration of amino acids was influenced by herbivory treatment, with higher concentrations in the absence of the AG herbivore. In the roots, total amino acid concentrations were highest in KIM when both the AG and BG herbivore were present. Johnson et al. (2009) examined amino acids in barley and found that BG herbivory by wireworms had little effect on amino acid concentrations in the leaves, whereas AG herbivory by aphids reduced amino acid concentrations. In tomatoes, changes in primary metabolites in response to herbivory are tissue- and herbivore-specific (Steinbrenner et al. 2011). In a field study with blackcurrant bushes, it was also found that BG herbivory by weevils increased foliar amino acid concentrations (Johnson et al. 2013). Thus far there has been no research on primary metabolites in wild cabbage in the context of AG-BG interactions. Cole (1997) looked at the effect of amino acids on the performance of the specialist aphid, *Brevicoryne brassicae*, and found that the intrinsic rate of increase was positively correlated with concentrations of amino acids in the phloem. The amino acid profiles in both the leaves and the roots were influenced by an interaction between plant populations and herbivory treatment. Our results, together with those of other studies, show that there is no general pattern in amino acid induction following BG herbivory.

Total sugar concentrations and sugar profiles in the leaves and roots did not differ between the wild cabbage populations, but were affected by herbivory regime. AG herbivory decreased total sugar concentrations in the leaves, whereas BG herbivory increased total sugar concentration in the roots. The reduced sugar concentration in the leaves could be the result of leaf damage and an associated reduction in photosynthesis, since sucrose is one of the end products of photosynthesis (Huber 1989). Re-allocation of resources from the damaged plant parts to undamaged plant parts as a reaction to leaf herbivory is a known phenomenon and part of a plant defence mechanism known as tolerance (Rosenthal and Kotanen 1994; Stowe et al. 2000; Orians et al. 2011). However, there was no effect of AG herbivory on root sugar concentrations, only more directly of BG herbivory. The higher root sugar concentrations following BG herbivory could have been the result of regrowth of roots and thus a higher demand for resources.

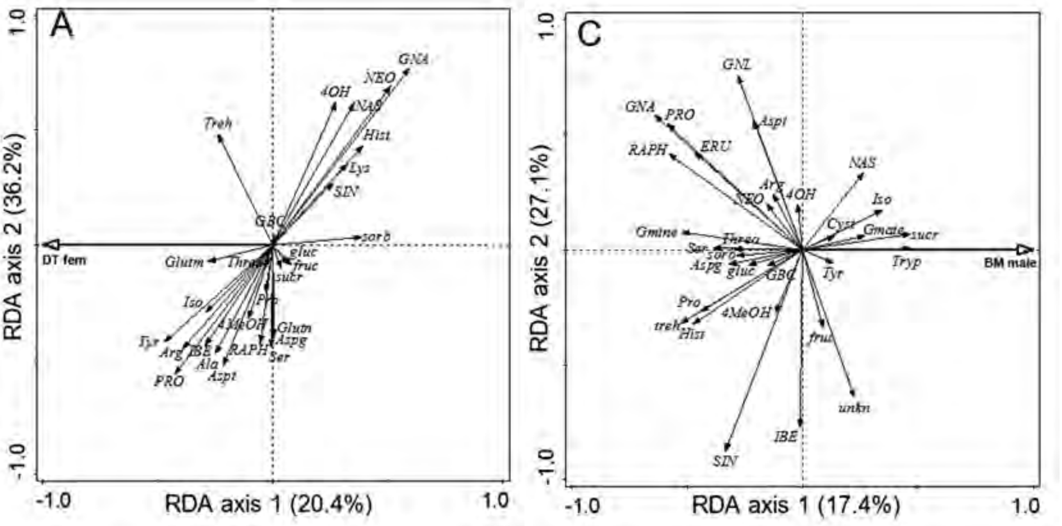
Linking insect performance and plant chemistry

Correlations between insect performance and chemistry variables were only significant in the presence of a herbivore in the opposite compartment. This means that only herbivory-induced variation could systematically explain some of the variation in the performance of the herbivore in the other compartment, whereas in the absence of a second herbivore, variation in phytochemicals did not lead to consistent patterns that could explain variation in insect performance. In general, the correlations between chemistry, both primary and secondary, and performance variables were negative. This means that high phytochemical concentrations correlated negatively with performance variables. There were some exceptions. For instance, high relative concentrations of the GS progoitrin, the amino acid tyrosine and the sugar trehalose positively correlated with performance variables of female *P. xylostella*.

Correlation analyses can only be used for explorative purposes. Here, results of these analyses showed that there were no clear systematic patterns that linked plant chemistry with insect performance, but also that there was considerable variation in the profiles of primary and secondary metabolites among the populations. The multivariate analysis used here gives equal weight to each of the compounds, whereas the biological-active concentration ranges are compound specific. Furthermore, this type of analysis ignores synergistic and antagonistic effects between chemicals. In addition, other phytochemicals that were not measured here may have affected insect performance.

In summary, our results highlight the fact that changes in plant quality in response to AG or BG herbivory or both can be relatively subtle and that the effects on the developmental performance of the specialized insects involved are species-specific with visible effects that may ‘jump’ from the plant to the third trophic level. Moreover, for the wild cabbage populations studied here, differences in plant quality between the populations appear to be greater than the herbivore-induced changes in plant quality. This does not mean that within-population variation is not present, but that the between-population variation masks effects mediated by AG and BG herbivores. Furthermore, given that they are all specialists, the insects are probably labile in terms of their ability to deal with differences in plant quality. Chemical analysis of leaf and root tissues showed that there is significant variation in primary and secondary chemistry between the populations and that the herbivore-induced changes are often tissue and population-specific. It may be that this variation has more apparent effects on less well adapted organisms, including generalist herbivores, as previous studies have shown (e.g. Gols et al. 2008b). Future work is needed to explore AG and BG interactions in wild cabbages across a broader range of insect species.

Supplementary material



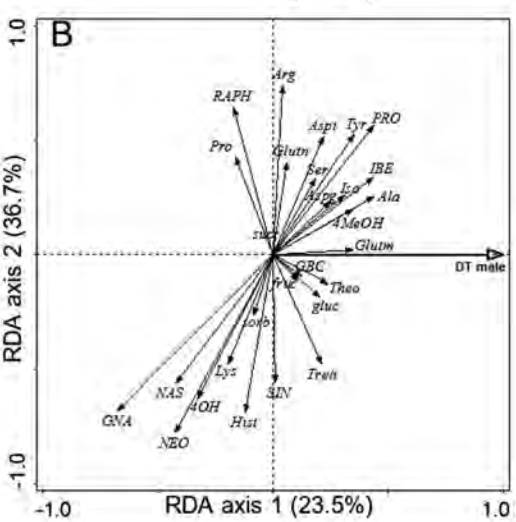


Figure S1 RDA ordination plots of the correlations between male adult body mass of *D. radicum* and chemistry (A), and between female (B) and male (C) development time of *P. xylostella* and chemistry. The percentage explained variation is given for each axis between parentheses. Abbreviations aliphatic GS: ALY= glucoalyssin, EUR = glucoerucin, GBN = glucobrassicanapin, GNA = gluconapin, GNL = gluconapoleiferin, PRO = progoitrin, RAPH = glucoraphanin, SIN = sinigrin; indole GS: 4MeOH = 4-methoxyglucobrassicin; 4OH = 4-hydroxyglucobrassicin, GBC = glucobrassicin, NEO = neoglucobrassicin; aromatic GS: NAS = gluconasturtiin; other GS: UnknSulf = unknown sulfide. Abbreviations amino acids: Ala = alanine, Arg = arginine, Aspg = asparigine, Aspt = aspartate, Cys = cystine, Glutm = glutamate, Glutn = glutamine, Hist = histidine, Iso = isoleucine, Lys = lysine, Pro = proline, Ser = serine, Threo = threonine, Tryp = tryptophan, Tyr = tyrosine. Abbreviations sugars: Fruc = fructose, Gluc = glucose, Sorb = sorbitol, Sucr = sucrose, Treh = trehalose

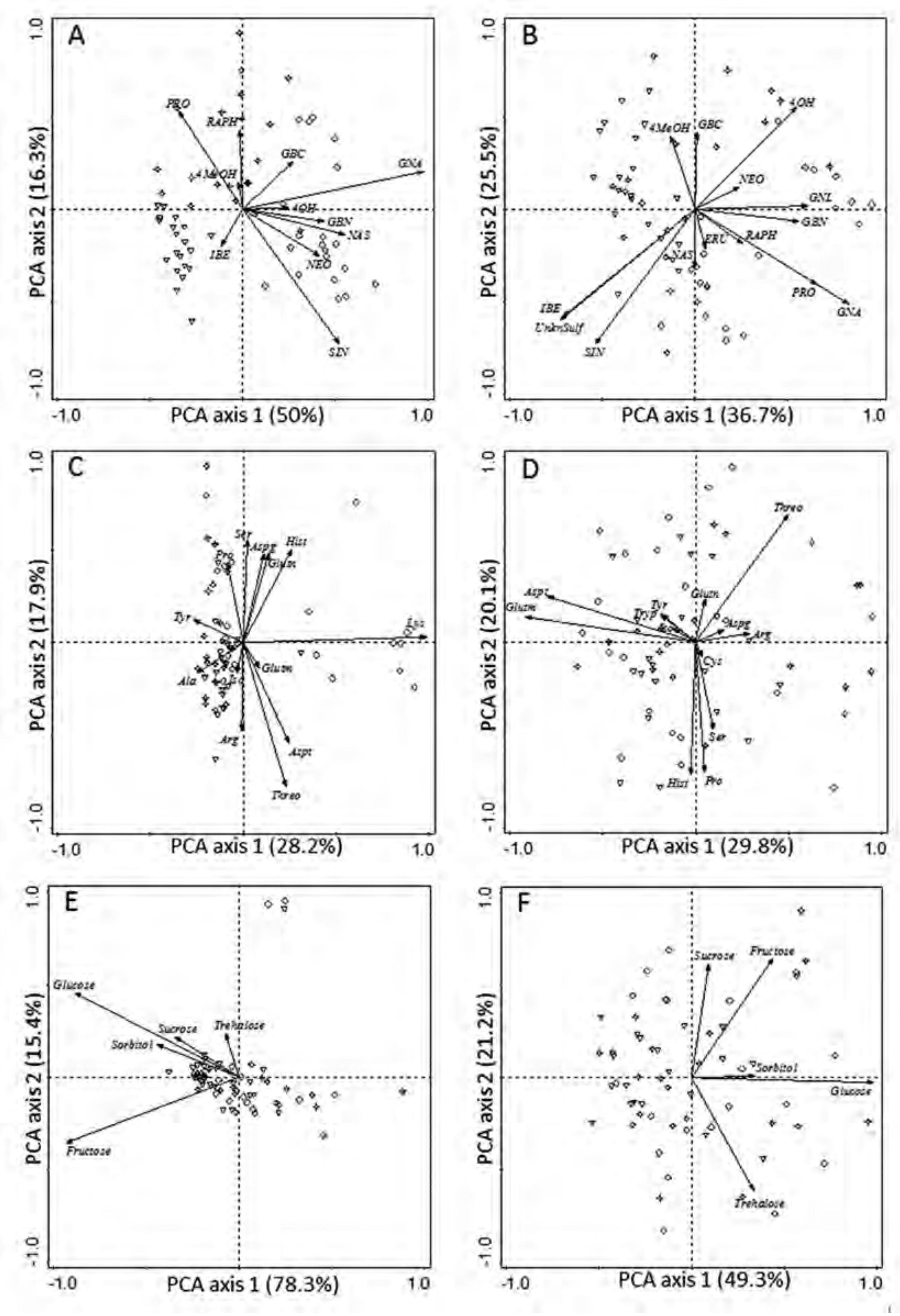

A
PCA axis 2 (16.3%)
PCA axis 1 (50%)
1.0
-1.0
-1.0
1.0
PRO
RAPH
4MeOH
GBC
GNA
4OH
GBN
NAS
NEO
IBE
SIN
B
PCA axis 2 (25.5%)
PCA axis 1 (36.7%)
4OH
4MeOH
GBC
NEO
GNL
GBN
ERU
RAPH
NAS
PRO
GNA
IBE
UnknSulf
SIN
C
PCA axis 2 (17.9%)
PCA axis 1 (28.2%)
Ser
Aspg
Hist
Pro
Glutm
Tyr
Lys
Glutm
Ala
Arg
Aspt
Treo
D
PCA axis 2 (20.1%)
PCA axis 1 (29.8%)
Treo
Aspt
Glutm
Glutm
Tyr
Tryp
Aspg
Arg
Cys
Ser
Hist
Pro
E
PCA axis 2 (15.4%)
PCA axis 1 (78.3%)
Glucose
Sucrose
Trehalose
Sorbitol
Fructose
F
PCA axis 2 (21.2%)
PCA axis 1 (49.3%)
Sucrose
Fructose
Sorbitol
Glucose
Trehalose

Figure S2 RDA ordination plots of the GS profiles for leaves (A) and roots (B), the amino acid profiles for leaves (C) and roots (D) and the sugar profiles for leaves (E) and roots (F) of the three wild cabbage populations. The percentage explained variation is given for each axis between parentheses. Populations: KIM = downward triangle, WIN = diamond, OH = star. Abbreviations aliphatic GS: ALY= glucoalyssin, EUR = glucoerucin, GBN = glucobrassicanapin, GNA = gluconapin, GNL = gluconapoleiferin, PRO = progoitrin, RAPH = glucoraphanin, SIN = sinigrin; indole GS: 4MeOH = 4-methoxyglucobrassicin; 4OH = 4-hydroxyglucobrassicin, GBC = glucobrassicin, NEO = neoglucobrassicin; aromatic GS: NAS = gluconasturtiin; other GS: UnknSulf = unknown sulfide. Abbreviations amino acids: Ala = alanine, Arg = arginine, Aspg = asparigine, Aspt = aspartate, Cys = cysteine, Glutm = glutamate, Glutn = glutamine, Hist = histidine, Iso = isoleucine, Lys = lysine, Pro = proline, Ser = serine, Threo = threonine, Tryp = tryptophan, Tyr = tyrosine. Abbreviations sugars: Fruc = fructose, Gluc = glucose, Sorb = sorbitol, Sucr = sucrose, Treh = trehalose

Chapter 6

General Discussion

Introduction

Interactions between plants and insects have been the subject of studies for many decades and cover a wide range of themes, from those involving mutualisms such as pollination to those involving antagonisms such as herbivory. Focusing on herbivory, plant and insects are often involved in co-evolutionary arms races in which plants evolve to defend themselves against herbivore attack, whereas insects evolve mechanisms to overcome these defences (Ehrlich and Raven 1964). Early studies on plant-insect interactions focused on bi-trophic interactions, i.e. interactions between plants and their herbivores. Later, inspired by a seminal paper by Price et al. (1980), third trophic levels were also incorporated into this field of research.

When studying plant-insect interactions, it is important to keep in mind that plants reside in both air and soil at the same time. This means that when plants are attacked in either compartment, this may have consequences for defence strategies in both compartments. Organisms in opposite compartments can influence each other indirectly through changes in plant primary and secondary chemistry, which together determine the food quality of plants for herbivores. In nature, plants display genetic variation in many traits including defence chemistry. Plant chemical defence is assumed to be costly to maintain (Agrawal et al. 2002; Strauss et al. 2002), and thus there is often a trade-off between putting energy to growth and reproduction, and to defence (Stearns 1992). Natural variation in plant traits is a prerequisite for evolution, which is shaped by differences in biotic and abiotic selection pressures acting on heritable genetic variation in plant traits (Whitham et al. 2003; Agrawal 2004; Lankau 2007; Hughes et al. 2008).

The main aim of my thesis was to study if genetic variation in plant chemistry affects (multi)trophic above- and belowground interactions (hereafter AG-BG interactions). I performed a number of experiments to elucidate some aspects concerning genetic variation in plant chemistry and plant-insect interactions. In this discussion I will first focus on plant-insect interactions in the aboveground compartment (chapters 3 and 5), followed by interactions belowground (chapters 4 and 5) and then by interactions between the above- and belowground compartments (chapter 5). After that I will discuss primary and secondary chemistry in the context of insect performance (chapter 4 and 5). Finally, I will conclude the discussion and identify a few topics for future research.

Aboveground plant-insect interactions

Interactions between plants and insects in the aboveground compartment have been well studied (Ehrlich and Raven 1964; Benson et al. 1975; Jaenike 1990; Mitter et al.

1991; Berenbaum and Zangerl 1998; Awmack and Leather 2002; Agrawal et al. 2006; Ali and Agrawal 2012). The outcomes of these interactions are diverse and depend on a number of factors, amongst others on how well the insect herbivore is adapted to overcome the defence of a plant. This may have consequences for species in the third trophic level. Both bi-trophic and multi-trophic aboveground interactions were studied in this thesis.

Bi-trophic interactions

Development time and adult body mass of a specialist herbivore, *Plutella xylostella*, differed between three wild cabbage populations and displayed a trade-off between these fitness parameters (**chapter 5**). The moths developed fastest on Winspit plants but also had lowest adult body mass on that population, and vice versa on Kimmeridge plants. Wild cabbage populations have also been show to differentially affect the performance of the specialist *Pieris rapae* in terms of development time and pupal mass, and survival of the generalist *Mamestra brassicae* (Gols et al. 2008b). Similar to *P. xylostella*, the development time of *P. rapae* was long on Kimmeridge (Gols et al. 2008b), which for *P. rapae* corresponded with high concentrations of the indole glucosinolate neoglucobrassicin. Reduced survival of *M. brassicae* corresponded with high concentrations of the aliphatic glucosinolate gluconapin and sinigrin (Gols et al. 2008b). For *P. xylostella* I found significant correlations between female and male development time and plant primary and secondary chemistry, but only when the root herbivore was present. For the females higher concentrations of progoitrin were correlated with a longer development time, whereas for the males this correlation was found for glucoiberin (**chapter 5**).

Herbivorous insect species are thus differently affected by individual glucosinolate compounds, revealing that genetic variation in the defence chemistry of wild cabbage plants will have varying consequences for herbivorous insects. This effect of genetic variation in plant chemistry on herbivorous insects is of course not restricted to wild cabbage, but applies to every plant community/population/species that exhibits naturally occurring genetic variation in their chemistry (Agrawal et al. 2006; Crutsinger et al. 2006).

Multi-trophic interactions

In **chapter 3** I explored the generally accepted theory that plant allelochemical sequestration acts as a defence mechanism (Winde and Wittstock 2011) by using a generalist predator (*Podisus maculiventris*) and a sequestering herbivore (*Athalia rosae*) as its prey. Although previous studies with *A. rosae* have provided evidence that easy bleeding (i.e. the release of a drop of haemolymph through a voluntary rupture of the larva's integument) deters predators (Müller et al. 2002; Müller and Brakefield 2003), I found that *P. maculiventris* was not deterred by easy bleeding, and that the performance of *P. maculiventris* was only marginally affected by *A. rosae*.

Larvae of *A. rosae* selectively sequester glucosinolates from their host plant, but they do not produce their own myrosinase. The glucosinolates-myrosinase (GS-MYS) system is the chemical defence mechanism of wild cabbage plants (Bones and Rossiter 1996; Rask et al. 2000; Fahey et al. 2001; Mithen 2001; Renwick 2002; Halkier and Gershenzon 2006; Textor and Gershenzon 2009). With one important component of the GS-MYS system missing within the body tissues of *A. rosae*, this may explain why feeding on *A. rosae* did not negatively affect the performance of the generalist predator *P. maculiventris*.

Other species, such as the cabbage aphid *Brevicoryne brassicae*, do produce their own myrosinase and are thus able to use the GS-MYS system for their defence (Winde and Wittstock 2011). For example, Francis and colleagues (2000) investigated the effect of glucosinolate sequestration by aphids on the performance of a generalist predator, the lady beetle *Adalia bipunctata,* when reared on three different crucifer species. When reared on plants with high glucosinolate concentrations, the specialist aphid *B. brassicae*, which sequesters glucosinolates, was more toxic then the generalist aphid *Myzus persicae*, which excretes glucosinolates in its honeydew (Francis et al. 2001). In the body tissue of *B. brassicae* not only glucosinolates but also their breakdown products, isothiocyanates, were detected, which was not the case for *M. persicae*. A study by Kos et al. (2011) showed that selective sequestration of glucosinolates by *B. brassicae* negatively affected the performance of two generalist predators. The food plant of *B. brassicae* affected its glucosinolates profiles, illustrating that plant chemistry affects predators via their prey (Kos et al. 2011). I conclude that sequestration in some herbivores like *A. roseae* is an alternative way of harmlessly dealing with plant secondary metabolites.

In **chapter 5** I studied the development of the endoparasitoid *Cotesia vestalis* and found that its performance in terms of survival and development time was affected by an interaction between plant population and type of herbivory (e.g. above- or belowground). In other words, the effect of belowground herbivory depended on the wild cabbage population upon which the endoparasitoid's host was reared. A study by Harvey et al. (2003) looking at the performance of both the endoparasitoid *Cotesia glomerata* and the hyperparasitoid *Lysibia nana* showed that the performance of the endoparasitoid was not affected by the food plant of its host (*Pieris brassicae*). In contrast, the performance of the hyperparasitoid was affected by the food plant of the herbivore: better performance on *B. oleracea* than on *B. nigra*, of which the latter had 3-5 times higher concentrations of glucosinolates in young shoots (Harvey et al. 2003). Other studies have also found that the quality of food plants for herbivores affect the performance of parasitoids (Barbosa et al. 1986; Kester and Barbosa 1994; Sznajder and Harvey 2003; Harvey et al. 2005; Coley et al. 2006; Ode 2006; Gols et al. 2008b; Lampert et al. 2008). These studies show that plant quality for herbivores can affect the performance of higher trophic levels. By influencing the body tissue of herbivores (both quantitative and qualitative), plant chemistry affects the quality of herbivores as hosts or prey for parasitoids and predators, respectively. This effect can cascade up to even the fourth trophic level (Harvey et al. 2003).

Belowground plant-insect interactions

The cabbage root fly, *Delia radicum*, is a specialist herbivore that uses shoot glucosinolates as cues for oviposition (Roessingh and Stadler 1990). The larvae of this species feed on root tissues close to the soil surface. Most studies using *D. radicum* as a belowground herbivore focused on the induction of defence chemistry and effects on the performance of aboveground trophic levels (Soler et al. 2005; van Dam et al. 2005; van Dam and Raaijmakers 2006). However, some studies have also looked at the performance of *D. radicum* in response to aboveground herbivory (Soler et al. 2007a; **chapter 5**) or different plant genotypes (van Leur et al. 2008; van Geem et al. 2015).

Van Leur et al. (2008) studied the performance of *D. radicum* on two chemotypes of *Barbarea vulgaris* that differed in their glucosinolate profiles and found that the pupal mass of *D. radicum* was higher on the chemotype that had glucobarbarin as its dominant compound. I found that the performance of *D. radicum* did not differ between the five genetically different wild cabbage populations, nor did I find a correlation between plant chemistry and root fly performance (**chapter 4**). This would suggest that *D. radicum* is well adapted to the wide range of total concentrations and specific glucosinolates compounds displayed by the wild cabbage populations. However, given that mortality of the root fly was high in all populations, it might be that differences in plant quality were masked by the variation in survival, as was also found in the Soler et al. (2007) study. It is difficult to monitor the development of soil organisms, and especially the stage at which mortality occurred.

Aboveground - belowground interactions

How do interactions between above- and belowground herbivores affect their performance? Through elicited changes in plant primary and secondary chemistry, herbivores in one compartment can affect herbivores in the opposite compartment (Gange and Brown 1989; Bardgett et al. 1998; Blossey and Hunt-Joshi 2003; van Dam and Heil 2011; Johnson et al. 2013). Since changes in plant chemistry in response to herbivory can differ between genotypes of a plant species, the outcome of AG-BG interactions is –at least partly– affected by plant genotype (Lampert et al. 2011).

Bitrophic interactions

Early studies on AG-BG interactions found positive effects of belowground herbivory on the performance of aboveground insects via improved food quality of plants because of increased nutrient availability (Gange and Brown 1989; Masters et al. 2001). However, Soler et al. (2005) found a negative effect of belowground herbivory by *D. radicum* on the performance of an aboveground herbivore and higher trophic levels associated with it. The development time of the herbivore, *P. brassicae*, increased on plants with belowground herbivory. A similar negative effect of *D. radicum* herbivory on *P. rapae* performance was found by van Dam et al. (van Dam et al. 2005). Both studies linked this negative effect to increased concentrations of secondary metabolites that decreased food quality for *P. rapae* caterpillars. In my thesis research, belowground herbivory by *D. radicum* did not affect the performance of the aboveground herbivore *P. xylostella*, whereas by contrast there were plant population-related effects (**chapter 5**).

Both *P. rapae* and *P. xylostella* are specialist herbivores adapted to feed on cruciferous plants. The observed dissimilarities in the effect of BG herbivory on AG performance can thus not be attributed to dietary breadth (generalists are usually more affected by qualitative and quantitative variations in plant defence chemistry; Schoonhoven et al. 2005). However, the plant species used in the experiments were quite different: whereas Soler et al. (2005) and van Dam et al. (2005) worked with an annual species, *B. nigra*, I worked with a perennial species, *B. oleracea*. The defence chemistry of *B. nigra* is dominated by one compound, namely sinigrin, which is not the case for *B. oleracea* where several compounds can be present in relatively high concentrations. Also, I used multiple populations of *B. oleracea* that are chemically different from each other, whereas the *B. nigra* plants were obtained from a single Dutch population (Soler et al. 2005; van Dam et al. 2005). Variation in defence chemistry between the wild cabbage populations was greater than variation in defence chemistry induced by belowground herbivory, which implies that any effects of belowground herbivory on the performance of *P. xylostella* were masked by the variation among the populations (**chapter 5**). It is evident that not only the identity of the belowground herbivore is important in determining the outcome of AG-BG interactions, but also the plant species, making these studies highly context-dependent.

Multi-trophic interactions

Multi-trophic interactions can also be affected by interactions between insects in the above- and belowground compartments. Belowground herbivory, depending on plant population, affected the performance of the parasitic wasp, *Cotesia vestalis*, with *P. xylostella* as its host (**chapter 5**). Other studies have also shown that belowground processes, including herbivory, affect the performance of higher trophic levels aboveground, either positively (Johnson et al. 2011) or negatively (Bezemer et al. 2005), whereas other studies reported no discernible effects (Wurst and Jones 2003).

Although I did not study the effect of aboveground herbivory on a third trophic level belowground in this thesis, other researchers have looked at such interactions with *D. radicum* as the belowground host for a parasitoid. For instance, Soler et al. (2007a) found that aboveground herbivory negatively affected the performance of *D. radicum* and its parasitoid *Trybliographa rapae* by reducing survival by more than 50% and also reducing adult body mass. Aboveground herbivory increased concentrations of indole glucosinolates in the roots, suggesting that the lowered performance of the root fly could have been, at least partly, caused by high levels of these secondary metabolites (Soler et al. 2007a). Aboveground herbivory by *P. brassicae* also negatively affected host plant location by the parasitoid *T. rapae*, reducing attraction to host-infected plants and also reducing field parasitism levels (Pierre et al. 2011).

Knowledge on how plant quality affects the natural enemies of herbivores is especially important in the field of biological control. For an effective top-down control of herbivores, it is important that negative effects of plant chemistry on natural enemies is properly assessed (Ode 2006). A natural enemy is an inefficient biological agent when its performance is negatively affected by plant chemistry as mediated through its host. This conflict is rarely addressed in studies of indirect plant defences, which is a major oversight.

Primary and secondary chemistry

One of the aims of my thesis was to find out how population-related differences in primary and secondary chemistry affect the performance of above- and belowground herbivores and higher trophic levels. In several experiments (**chapters 4** and **5**) I recorded both insect performance and measured plant chemistry, and analysed the data for correlations between plant chemistry and insect performance. I found correlations between male adult body mass of *D. radicum* and plant chemistry when the aboveground herbivore was absent. Also, there were correlations between female and male development time of *P. xylostella* and plant chemistry, but only when the belowground herbivore was present. Thus, the presence or absence of herbivores in the opposite compartments differentially affected the influence of plant chemistry on the performance of *D. radicum* and *P. xylostella*.

With respect to secondary chemistry, the fact that glucosinolates did not have a strong and unidirectional negative effect on the insects suggests that the insect species used in the experiments, being specialists on Brassicaceous species, are well adapted to a broad range of glucosinolate concentrations (both total and of individual compounds). I also did not find strong links between insect performance and plant primary chemistry. Only male adult body mass of *D. radicum* was positively correlated with a few amino acids. This suggests that compounds other than amino acids and carbohydrates played a role in the observed differences in insect performance among the wild cabbage populations. Nitrogen for example is an important determinant of plant quality for herbivores and is often limiting (Mattson 1980; Awmack and Leather 2002; Fagan et al. 2002; Coley et al. 2006). Analysing the chemistry of wild cabbage plants with regard to elements such as nitrogen and carbon could provide important additional insights into the quality of these plants for herbivores.

Concluding remarks and ideas for future research

AG-BG interactions have been the subject of many studies, of which most studies focused on only one genotype of a plant species. The results of such studies have enhanced mechanistic understanding, but they do not account for the variation in plant traits, such as morphology, phenology and chemistry, as they occur in nature. With regard to plant defence chemistry, it has remained unclear how this genetic variation affects herbivorous insects and their natural enemies, or what the consequences are for AG-BG interactions. In this thesis I have shown that population-related genetic variation in plant chemistry has no unidirectional effect on plant-insect interactions. The outcome of (multi)trophic interactions between plants and insects depended on the species involved and there were no clear correlations between plant chemistry and insect performance. Often there was an interaction between wild cabbage population and type of herbivory as explanatory variables, suggesting that complex plant-insect interactions are influenced by multiple factors simultaneously.

In my experiments I incorporated the aspect of plant population-related genetic variation in plant chemistry into the research field of (multi)trophic plant-insect interactions, both above- and belowground. However, there are a number of specific topics that have not been addressed yet and require further research. I highlight some main topics below.

- In this thesis I studied chewing herbivorous insects. However, herbivores that feed on the phloem of plants (e.g. aphids) may encounter different plant metabolites than those that chew on leaves and stems. Therefore, it is likely that the effects of genetic variation in plant chemistry on insects will depend on insect **feeding modes**. Feeding modes could affect the outcome of certain multi-trophic AG-BG interactions.

- The wild cabbage populations used in the present study have been extensively studied with regard to their aboveground associated invertebrates. Future research should focus on the **belowground compartment** in order to describe belowground communities and identify important species in the soil. This knowledge would contribute to the overall understanding of interactions between the wild cabbages and their associated communities.

- In **chapter 5** I studied the effect of belowground herbivory on an aboveground trophic chain. In nature, the belowground herbivore *D. radicum* harbours natural enemies such as the parasitoid *Trybliographa raphae*. It is still unclear whether including a **third trophic level organism belowground** would have consequences for the responses of aboveground (multi)trophic interactions to belowground interactions.

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Summary
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Summary

Plants form the basis of many food webs and are consumed by a wide variety of organisms, including herbivorous insects. Over the course of evolution, plants have evolved mechanisms to defend themselves against herbivory, whereas herbivorous insects have evolved counter-mechanisms to overcome these defences (a.k.a. co-evolutionary arms races). Plant-insect interactions are not restricted to plants and their herbivores (bi-trophic interactions), but also involve natural enemies of the herbivores such as parasitoids and predators (tri-trophic interactions). Plant quality can affect the quality of the host or prey for parasitoids and predators, respectively. In addition, other plant traits are important in providing shelter, alternative food sources, or chemical cues that can be used for host/prey location. Moreover, as plants reside in both soil and air, they mediate interactions between organisms above- and belowground through changes in plant quality. Plant quality is determined by secondary metabolites and morphological traits that may negatively affect the performance of insects, as well as by primary metabolites that plants produce in order to grow, develop and reproduce, which also provide essential nutrients for insects.

Natural plant populations often exhibit genetic variation in various plant traits that include, amongst others, primary and secondary chemistry. Genetic variation in plant defence traits, such as the production of secondary metabolites, can be under selection pressure from a suite of biotic and abiotic factors that vary in space and time. Herbivorous insects may encounter a wide range of plant metabolites because the total concentrations of primary and secondary metabolites and the concentrations of individual compounds vary between genetically different plants. Also as a consequence of genetic variation, plants can respond differently to herbivory in terms of induced defence chemistry and re-allocation of metabolites.

The main aim of this thesis was to study how genetic variation in plant chemistry affects (multi)trophic interactions between wild cabbage plants and associated insects, both above- and belowground. As a model system I used five naturally occurring populations of wild cabbage (*Brassica oleracea*) located in the Dorset area in the UK. These populations have been shown to genetically differ in their defence chemistry profiles even though they are located in relatively close proximity to each other. Wild cabbages belong to the Brassicaceae, a plant family that is characterized by the production of glucosinolates, a group of secondary metabolites. Together with the enzyme myrosinase they form the chemical defence system of Brassicaceous plants including wild cabbage. Glucosinolates and myrosinases are stored separately in plant tissues but upon tissue damage they come into contact with each other upon which the glucosinolates are hydrolysed into potentially toxic break down products.

The wild cabbage populations used in this thesis differ in their total glucosinolate concentrations as well as in the expression of individual glucosinolates.

In **chapter 1** I describe plant-insect interactions in a multi-trophic framework, including both the above- and belowground compartments. Genetic variation in plant traits is introduced as the main topic of this thesis, and I present the main aim and outline of my work.

In **chapter 2** I discuss how aboveground-belowground interactions influence the evolution and maintenance of genetic variation in plant defence chemistry. I review literature on AG-BG interactions as selection pressures for genetic variation, discuss hypotheses about plant mediation of AG-BG interactions, identify gaps in our knowledge such as the influence of spatial-temporal variation in AG-BG interactions, and in the end present new data on genetic variation in secondary chemistry of wild cabbage and related species.

The co-evolutionary arms race between plants and insects has resulted in adaptations in herbivores to cope with plant defence traits. Some insect herbivore species concentrate or sequester secondary metabolites from their food plant and use them in defence against their own enemies. In **chapter 3** I studied whether sequestration of glucosinolates by a specialist herbivore is an effective defence mechanism against a generalist predatory bug. I used the sequestering herbivore *Athalia rosae* as one prey species, and the non-sequestering herbivore *Pieris rapae* as the control prey species. I compared the performance of the predatory stink bug *Podisus maculiventris* on these two prey species. As an extra factor, the two prey species were each reared on three different wild cabbage populations to test if plant population would have an effect on the predator through the sequestering herbivore. I found no consistent effect of plant population on the performance of the predator, and prey species only marginally affected its performance. Based on the results I suggest that in some trophic interactions sequestration is not an effective defence mechanism but merely an alternative way of harmlessly dealing with plant secondary metabolites.

In addition to aboveground plant-insect interactions, belowground interactions were considered as well. To test whether the performance of the belowground specialist herbivore *Delia radicum*, of which the larvae feed on root tissues, was influenced by population-related variation in defence chemistry, I reared this species on the five wild cabbage populations (**chapter 4**). Chemical analyses of root tissues revealed that there were differences amongst the populations in plant primary (amino acids and sugars) and secondary (glucosinolates) chemistry, but this did not affect the performance of the root herbivore, suggesting that *D. radicum* is well adapted to a wide range of total concentrations and concentrations of individual metabolites.

Whereas in **chapters 3** and **4** I only focused on one compartment (aboveground and belowground respectively), in **chapter 5** I included both compartments in one experiment. I studied the effect of belowground herbivory by larvae of the root fly *D. radicum* on the performance of an aboveground multi-trophic food chain, and whether this effect differed among three wild cabbage populations. I found that belowground herbivory differentially affected the performances of a specialist aboveground herbivore, the diamondback moth *Plutella xylostella*, and its parasitoid, *Cotesia vestalis*, with the parasitoid being more affected than the herbivore. Their performance also differed between the wild cabbage populations, often in interaction with the presence/absence of the belowground herbivore. For both the above- and belowground herbivore I found correlations between performance and plant chemistry, which differed between the insect species and also between males and females.

In **chapter 6** I discuss the results of my experiments in relation to other studies. I finish with a general conclusion about my work and provide some ideas for future studies that could contribute to our knowledge in the field of (multi)trophic above-belowground interactions with regard to genetic variation in plant chemistry.

In my thesis I show that genetic variation in plant chemistry can affect the outcome of above-belowground plant-insect interactions. Herbivores and higher trophic levels were differently affected by the wild cabbage populations, and this difference was also influenced by the location of herbivory (i.e. aboveground or belowground). In both **chapter 4** and **chapter 5** I found no strong, unidirectional links between plant chemistry and insect performance, suggesting that other metabolites may have played a role in the observed differential effects of the wild cabbage populations. I also show that sequestration of plant allelochemicals in some herbivores is an alternative way of harmlessly dealing with plant secondary metabolites instead of an effective defence mechanism against predators (**chapter 3**).

Samenvatting

Planten vormen de basis van veel voedselwebben en worden geconsumeerd door een grote verscheidenheid aan organismen, waaronder plantenetende insecten. In de loop der evolutie hebben planten mechanismen ontwikkeld om zich te verdedigen tegen herbivorie, terwijl plantenetende insecten tegen-mechanismen hebben ontwikkeld om zulke verdedigingsmechanismen te overwinnen (= co-evolutionaire wapenwedloop). Interacties tussen planten en insecten zijn niet beperkt tot planten en hun herbivoren (bi-trofische interacties), maar omvatten ook natuurlijke vijanden van de herbivoren zoals sluipwespen en predatoren (tri-trofische interacties). De kwaliteit van een plant kan de kwaliteit van de gastheer of prooi voor respectievelijk sluipwespen en predatoren beïnvloeden. Verder zijn andere eigenschappen van planten belangrijk in het verschaffen van schuilplaatsen, alternatieve voedselbronnen, of chemische aanwijzingen die gebruikt kunnen worden voor het localiseren van gastheer/prooi. Daarnaast, aangezien planten zowel in de grond als in de lucht groeien, bemiddelen ze interacties tussen organismen boven en onder de grond via veranderingen in de kwaliteit van de plant. De kwaliteit van een plant wordt bepaald door secondaire metabolieten en morfologische eigenschappen die de ontwikkeling van insecten negatief kunnen beïnvloeden, maar ook door primaire metabolieten die geproduceerd worden door planten voor groei, ontwikkeling en voortplanting en die ook essentiele nutriënten voor insecten verschaffen.

Natuurlijke plantpopulaties leggen vaak genetische variatie in verschillende planteigenschappen aan de dag, waaronder primaire en secondaire chemie. Genetische variatie in de verdedigingseigenschappen van planten, zoals de productie van secondaire metabolieten, kan onder selectiedruk staan van een aantal biotische en abiotische factoren die variëren in ruimte en tijd. Plantenetende insecten kunnen een breed scala aan plant metabolieten tegenkomen omdat de totale concentraties van primaire en secondaire metabolieten en de concentraties van individuele metabolieten variëren tussen genetische verschillende planten. Een ander gevolg van genetische variatie is dat planten op verschillende manieren kunnen reageren op herbivorie in termen van geïnduceerde verdedigingschemie en herallocatie van metabolieten.

Het hoofddoel van dit proefschrift was om te bestuderen hoe genetische variatie in plantenchemie multi-trofische interacties tussen wilde kool planten en geassocieerde insecten beïnvloedt, zowel boven- als ondergronds. Als modelsysteem gebruikte ik vijf wilde kool (*Brassica oleracea*) populaties die van nature voorkomen in de Dorset regio in het Verenigd Koninkrijk. Van deze populaties is al aangetoond dat ze genetisch verschillen in het profiel van hun verdedigingschemie, ondanks hun relatief dichte nabijheid tot elkaar. Wilde kolen behoren tot de Brassicaceae, een plantenfamilie die gekarakteriseerd wordt door de productie van glucosinolaten, een groep van

secondaire metabolieten. Samen met het enzym myrosinase vormen ze het chemische verdedigingssysteem van Brassica-planten waaronder wilde kool. Glucosinolaten en myrosinasen zitten gescheiden van elkaar opgeslagen in plantenweefsel maar door weefselschade komen ze in contact met elkaar waarna de glucosinolaten gehydrolyseerd worden tot potentieel giftige afbraakproducten. De wilde kool planten gebruikt in dit proefschrift verschillen in hun totale glucosinolaat profielen maar ook in de expressie van individuele glucosinolaten.

In **hoofdstuk 1** beschrijf ik plant-insect interacties in een multi-trofische raamwerk, met inbegrip van zowel het bovengrondse als het ondergrondse compartiment. Genetische variatie in planteigenschappen wordt geïntroduceerd als het hoofdonderwerp van dit proefschrift, en ik presenteer het hoofddoel en de opzet van mijn werk.

In **hoofdstuk 2** bediscussieer ik hoe bovengrondse-ondergrondse interacties de evolutie en behoud van genetische variatie in de verdedigingschemie van planten beïnvloedt. Ik bespreek literatuur over bovengrondse-ondergrondse interacties als selectiedruk voor genetische variatie, identificeer gaten in onze kennis zoals de invloed van spatio-temporele variatie in bovengrondse-ondergrondse interacties, en aan het eind presenteer ik nieuwe data over genetische variatie in secondaire chemie van wilde kool en gerelateerde plantensoorten.

De co-evolutionaire wapenwedloop tussen planten en insecten heeft geresulteerd in aanpassingen in herbivoren om te kunnen omgaan met verdedigingseigenschappen van planten. Sommige plantenetende insectsoorten concentreren of slaan secondaire metabolieten van hun voedselplant op en gebruiken ze ter verdediging tegen hun eigen vijanden. **In hoofdstuk 3** bestudeer ik of het opslaan van glucosinolaten door een specialistische herbivoor een effectieve verdediging is tegen een generalistische roofwants. Ik gebruikte *Athalia rosae*, een herbivoor die metabolieten opslaat, als een prooisoort, en *Pieris rapae*, een herbivoor die niet opslaat, als de controle prooisoort. Ik vergeleek de ontwikkeling van de roofwants *Podisus maculiventris* tussen de twee prooisoorten. Als een extra factor waren de twee prooisoorten elk op drie verschillende wilde kool populaties opgekweekt om te testen of plantpopulatie een effect had op de predator via *A. rosae*. Ik vond geen consequent effect van plantpopulatie op de groei van de predator, en prooisoort beïnvloedde de groei slechts marginaal. Afgaande op de resultaten suggereer ik dat in sommige trofische interacties het opslaan van metabolieten geen effectieve verdediging is maar slechts een alternatieve manier om met secondaire plantmetabolieten om te gaan zonder daarvan negatieve effecten te ondervinden.

Naast bovengrondse plant-insect interacties werden ook ondergrondse interacties behandeld. Om te testen of de ontwikkeling van de ondergrondse specialistische herbivoor *Delia radicum*, waarvan de larven zich voeden met wortelweefsel, beïnvloed werd door populatie-gerelateerde variatie in verdedigingschemie, kweekte ik deze soort op de vijf wilde kool populaties (**hoofdstuk 4**). Chemische analyse van wortelweefsel liet zien dat er verschillen waren tussen de populaties in primaire (aminozuren en suikers) en secondaire (glucosinolaten) plantenchemie, maar dat dit geen invloed had op de ontwikkeling van de wortelherbivoor, wat suggereert dat *D. radicum* goed is aangepast aan een breed scala van totale concentraties en concentraties van individuele metabolieten.

Terwijl ik in **hoofstukken 3** en **4** enkel focuste op één compartiment (bovengronds en ondergronds respectievelijk), heb ik in hoofdstuk 5 beide compartimenten in één experiment bestudeerd. Ik onderzocht het effect van ondergrondse herbivorie door larven van de wortelvlieg *D. radicum* op de ontwikkeling van een bovengrondse multitrofische voedselketen en of dit effect verschilde tussen drie wilde kool populaties. Ik vond dat ondergrondse herbivorie de groei van een specialistische bovengrondse herbivoor, de koolmot *Plutella xylostella*, en haar sluipwesp, *Cotesia vestalis*, op verschillende manieren beïnvloedde, waarbij de sluipwesp meer beïnvloed werd dan de herbivoor. Hun ontwikkeling verschilde ook tussen de wilde kool populaties, vaak in interactie met de aan- of afwezigheid van de ondergrondse herbivoor. Voor zowel de boven- als ondergrondse herbivoor vond ik correlaties tussen hun groei en plantenchemie, welke verschilden tussen de insect soorten en ook tussen mannetjes en vrouwtjes.

In **hoofdstuk 6** bediscussieer ik de resultaten van mijn experimenten in relatie tot andere studies. Ik eindig met een algemene conclusie met betrekking tot mijn werk en geef enkele suggesties voor toekomstige studies die bij zouden dragen aan onze kennis in het veld van (multi)trofische boven-ondergrondse interacties met betrekking tot genetische variatie in plantenchemie.

In mijn proefschrift laat ik zien dat genetische variatie in plantenchemie de uitkomst van boven-ondergrondse plant-insect interacties kan beïnvloeden. Herbivoren en hogere trofische niveaus werden op verschillende manieren beïnvloed door de wilde kool populaties, en dit verschil werd ook beïnvloed door de locatie van de herbivoor (m.a.w. bovengronds of ondergronds). In zowel **hoofdstuk 4** als **5** vond ik geen sterke, unidirectionele relaties tussen plantenchemie en insectengroei, wat suggereert dat andere metabolieten wellicht een rol speelden in de geobserveerde verschillende effecten van de wilde kool populaties. Ik laat ook zien dat het opslaan van secondaire metabolieten in sommige herbivoren een alternatieve manier is om om te gaan met secondaire plantmetabolieten zonder nadelige gevolgen, in plaats van een effectief verdedigingssysteem tegen predatoren (**hoofdstuk 3**).

Acknowledgements

Four years may sound like a long time, but every PhD candidate preparing her/his dissertation for printing can tell you that they are gone before you know it. My PhD has been a rollercoaster ride and in these acknowledgements I would like to thank the people who made the high peaks possible, but also the ones that made the lows more bearable.

First I have to thank my supervisors **Jeff Harvey** and **Rieta Gols**. Thank you both for letting me be my independent self, but also for giving me a push when needed. I had a great time with you in the field in the UK where we sampled soil and roots during two consecutive summers. I will never forget the beautiful scenery, the crazy British people lying on the beach in Durdle Door when it was only 15 °C, the guy with the kite that ended up in the ocean, or all the people stopping to ask what we were doing and then have Jeff explaining it to them with great enthusiasm.

Rieta, I am grateful for your statistical knowledge and guidance, and for your rational thinking. You are a strong woman and I admire you for that. Thank you for always being available for questions or meetings, and also for emotional support in difficult times.

Jeff, thank you, not only for your extensive knowledge of ecology, insects, biology, birds, politics and what not, but also because of your jokes and good sense of humour. It has been great to have a supervisor to talk with about common interests such as music, animals, and the end of the world of course.

Olga and **Martine**, my dear paranymphs, roomies and friends, it was great sharing an office with you and having so much fun together. You guys were there for me when I had questions about work, but also when I needed a shoulder to cry on. We have been through a lot together, and I will cherish the memories forever. I don’t think I would have made it without your encouraging words and support!

Olga, you are such a dedicated scientist, I really admire that. You work hard and are eager to educate yourself about statistics and other topics. Thank you for your help and advice on many occasions. You are not just a great colleague, but also a sweet friend, always looking out for me and ready with a motivational speech. I know that sometimes you doubted whether your words connected in my stubborn head, but they did. So thank you for not giving up on me.

Martine, I love your positive energy and the fact that we have the same awesome sense of humour ;-) The huge amount of work that you did during your PhD was somewhat intimidating, but also inspiring. I enjoyed going to York together and finding the best vegan burger ever at Goji Café! Thank you for employing a push-and-pull strategy to make sure I finished my PhD.

Dear **Minghui**, thank you for your loyal friendship. We started and finished our PhD's around the same time and it was great to have someone beside me during those four years. You have come so far since the first day that we met in the phytotron, don't you ever forget that! Believe in yourself, because you deserve it. We have become close friends and I am happy to have such a warm and optimistic person in my life (who also happens to be a great cook). Can't wait to see you again, and I am definitely going to visit you in China!

Henk, het was altijd gezellig met jou :) Samen lunchen, buiten wandelen als het weer het toeliet, of de stad in om vegan eten uit te proberen. Die lunches houden we er gewoon in, al zal het wat minder frequent zijn. Van de zomer komen Martine en ik eindelijk je huis bewonderen! Natuurlijk ook bedankt voor het uitvoeren van analyses in het lab.

There are many more (ex)colleagues at the TE-department that I need to thank.

Wim, my promotor and head of the TE-department, thank you for your amazingly quick feedback on manuscripts, your inspirational out-of-the-box-thinking, and also for reminding me to take a step back sometimes to look at my work from a broader perspective. I'm also thankful for your help in getting my reading version finished in such a short time span!

Stijn and **Minggang**, thank you guys for being so funny, intentionally or not. **Jasper**, you were always ready to either listen or make me laugh, much appreciated! **Roeland**, I am not sure what you are up to right now but I am sure you are following your heart and that is good! **Tanja**, thank you for bringing so much joy to our office while you were still working at the NIOO.

Roxina, I am glad that we shared an office during the beginning of my PhD. The work that you did during your PhD served as an example for me. I could always talk to you or ask you a question and get good advice in return!

Without the efforts of **Roel** and **Gregor**, I couldn't have done my experiments. Thank you both for keeping things organized and smooth-running in the phytotron. **Ciska**, I appreciate all the help, advice and guidance that you gave me during my PhD with respect to chemical analyses in the lab. You are a good teacher and fun company.

The NIOO is a great working place, especially the TE-department. Thanks to all the scientists for creating such a warm and open working environment. Best of luck to all the current PhD students and also to the postdocs who are trying to find and create their place in science.

Natuurlijk wil ik ook mijn familie bedanken. Mam en pap, bedankt voor jullie onvoorwaardelijke steun in alles wat ik doe (hoe gek en impulsief het ook lijkt in eerste instantie) en jullie bemoedigende woorden. Bedankt dat jullie de WUR en de studie Bos- en Natuurbeheer bij mij onder de aandacht brachten toen ik zoekende was; voor het doorlezen van mijn manuscripten; voor jullie hulp elke keer dat ik binnen Wageningen verhuisde in de 11 jaar dat ik er heb gewoond; en voor het oppassen op de poezen wanneer ik naar het buitenland ging.

Mar, je bent niet alleen mijn zus maar ook mijn beste vriendin. Bedankt dat ik je altijd kon bellen voor advies of als ik even frustraties moest spuien. Altijd handig om feedback te krijgen van iemand buiten de wetenschap.

Jeroen, bedankt voor alle keren dat ik bij jou terecht kon met computerproblemen en gerelateerde vragen. Fijn dat je me altijd hebt geholpen en zo veel frustratie hebt bespaard!

I don't think I could have handled the many hours of weighing insects or entering data as well as I did without musical support. Spending evenings in the phytotron without The Rolling Stones to sing along with just isn't that much fun. I am grateful for the uplifting/ beautiful/ inspiring/ cool music produced by not only The Rolling Stones, but also Creedence Clearwater Revival, Guns n' Roses (original band formation!), Nirvana, Metallica, Lenny Kravitz, P!nk, Aerosmith, Bon Jovi, U2, Anouk, Muse, The Eagles, Ugly Kid Joe, Queen, and many other artists/bands. Rock on!

Tijdens een PhD wil je voorkomen dat je sociale leven inkakt, en gelukkig had ik veel mensen om mij heen die graag afleiding boden. **Evelien**, **Marina** en **Marcella**, bedankt voor alle gezellige avonden in Amsterdam! Fijn dat ik jullie na meer dan 15 jaar nog steeds in mijn leven heb. **Mariska**, heerlijk om een vriendin zo dicht bij Wageningen te hebben gehad die net zo gek is van dieren als ik :) **Valesca**, ik ben blij dat we elkaar hebben ontmoet in het asiel zoveel jaar geleden. Succes met je mooie toekomstplannen, ik heb er alle vertrouwen in! Lieve **Margriet**, bedankt dat je Ska zoveel liefde en goede zorgen hebt gegeven tijdens de veel te korte periode dat ze bij je was. Ik heb genoten van onze dagjes uit in Nijmegen. **Yasmijn**, ik heb een geweldige studietijd gehad mede dankzij jou! Altijd leuk om weer met je af te spreken en bij te kletsen. My vegan sister **Rodien**, ik zou willen dat we elkaar vaker zien want het is altijd supergezellig.

Lieve **José**, ik had het afronden van mijn PhD graag met jou erbij willen vieren, maar dat is helaas niet mogelijk. Jouw ziekte en overlijden zetten mijn leven op zijn kop en ik heb er lang over gedaan om het verlies een plek te kunnen geven. Je hebt mij doen inzien dat ik mijn passie moet volgen en niet genoegen nemen met minder. Het leven is te kort om niet je hart te volgen. Bedankt voor deze harde levensles...

Curriculum vitae

Moniek van Geem was born in Uithoorn, The Netherlands, on the 9th of March 1985. In 2003 she finished secondary school (atheneum) at Amstelveen College in Amstelveen. A year later she started the study Forest and Nature Conservation at Wageningen University (BSc) and graduated in 2007.

After working for one year she went back to university for a master in Forest and Nature Conservation, during which she specialized in animal ecology and did two theses. The first thesis brought her to South Africa, where she studied the foraging behaviour of goats at the University of KwaZulu-Natal in Pietermartizburg and fell in love with rugby. The second thesis brought her to the Netherlands Institute of Ecology (NIOO) at Heteren, where she studied the performance of a specialist herbivore, two primary parasitoids and two hyperparasitoids on different populations of black mustard plants. This thesis introduced her to the fascinating world of plant-insect interactions.

In 2010 she graduated with honours from Wageningen University, and half a year later she started her PhD at the department of Terrestrial Ecology at the NIOO, now located in Wageningen. Under supervision of Prof. Dr Jeffrey A. Harvey, Dr Rieta Gols and Prof. Dr Wim H. van der Putten she studied the effect of genetic variation in defence chemistry of wild cabbage on above- and belowground multi-trophic plant- insect interactions. During her PhD, Moniek presented her work at several (inter)national conferences, such as the European Congress of Entomology in York, UK (2014). The results of her PhD are presented in this thesis.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- The importance of aboveground-belowground interactions on the evolution and maintenance of variation in plant defence traits

Post-graduate courses (4.1 ECTS)

- Multivariate analysis; PE&RC (2012)
- Introduction to R for statistical analysis; PE&RC (2012)
- Soil ecology; PE&RC, SENSE, RSEE, Eco-Finders (2012)

Laboratory training and working visits (1.5 ECTS)

- Rearing of *Delia radicum*, the cabbage root fly; University of Rennes, France (2011)

Invited review of (unpublished) journal manuscript (1 ECTS)

- Ecological Entomology: AG-BG interactions on invasive plants (2014)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; PE&RC (2012)

Competence strengthening / skills courses (3.4 ECTS)

- Scientific publishing; WGS (2012)
- Competence assessment; Maas Assessment & Development (2012)
- Project and time management; Valley Consult (2012)
- Techniques for writing and presenting a scientific paper; WGS (2012)
- Reviewing a scientific paper; WGS (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)

- PE&RC PhD First years weekend (2011)
- PE&RC Day Biomimicry (2013)
- PE&RC Last years weekend (2014)
- WGS PhD Workshop carousel (2014)

Discussion groups / local seminars / other scientific meetings (9.2 ECTS)

- PhD Event KNAW (2011)
- NIOO PhD Monthly discussion meetings & seminars (2011-2015)
- NIOO Days (2013)
- 8th Plant-Insect workshop; Wageningen (2013)
- Current themes in ecology: the future of ecosystems, ecosystems of the future (2013)
- NERN-NAEM Meetings (2013, 2014)
- 9th Plant-Insect Workshop; Utrecht (2014)
- Current themes in ecology: biodiversity research at the crossroads (2014)

International symposia, workshops and conferences (6.9 ECTS)

- British Ecological Society (BES) annual meeting (2012)
- International Entomophagous Insects Conference (IEIC) 3 (2013)
- European Congress of Entomology (ECE) 10 (2014)

The research presented in this thesis was conducted at the department of Terrestrial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen. This is thesis 127.

The research described in this thesis was funded by the Netherlands Organization for Scientific Research (NWO) with a grant (821006) from the Earth and Life Sciences Foundation (ALW).

Cover art: Valesca Florijn
Wild cabbage illustration: Tat2addict (www.tat2addict.nl)
Design and Lay-out: AgileColor Design Studio/Atelier (www.agilecolor.com)
Printed by: GVO drukkers & vormgevers B.V. (www.gvo.nl)