Consequences of intra-specific metabolic diversity in plants for soil organisms

A baseline approach for evaluating ecological effects of genetic modifications

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Contents

SUMMARY

Plant intra-specific variation, *i.e.* variation within a plant species, is known to affect organisms that are directly associated to plants. These effects may be due to for example differences in nutritional quality or defensive metabolites. Plant intra-specific variation can also affect higher trophic level and organisms not directly associated to the plants, *i.e.* non-target organisms. These effects occur via differences in the quality of herbivores serving as host or prey, due to differences in the rates of attractiveness for higher trophic level organisms, differences in decomposition rates of litter, or differences in root exudates. Intra-specific variation occurs naturally in wild plant populations and humans have used this to select plants for agricultural use. Breeding and artificial selection for plant traits that were desirable for agricultural practices resulted in novel varieties, adding to intra-specific variation in these species.

In addition to natural variation and variation between cultivars, plant genetic modification can result in crop varieties with novel traits such as increased productivity or insect resistance. These traits may also affect non-target organisms. The question is whether these effects fall within or outside the range of non-target effects observed for conventionally bred varieties. To answer this question, one first needs to determine the range of non-target effects caused by conventional varieties, a so-called 'baseline'. In this thesis, I examined the baseline effects that conventional white cabbage varieties have on soil organisms, the mechanisms behind these effects, and the consequences for interactions between below-and aboveground organisms. The range of effects observed in the conventionally bred varieties can then serve as a baseline for evaluating the effects of genetic modifications.

I started by examining intra-specific variation in glucosinolate concentrations and profiles in white cabbage cultivars. Glucosinolates, a group of circa 120 secondary plant compounds predominantly present in the Brassicaceae, have been shown to affect not only aboveground organisms but also belowground organisms. Glucosinolate profiles in both roots and shoots of white cabbage cultivars showed significant intra-specific variation. The root glucosinolate profiles were more diverse than in the shoots. The variation in root glucosinolate profiles between four of the cultivars was used to evaluate

the effects of glucosinolates on a range of soil organisms from different trophic levels, which differ in their degree of association with plant roots. In the field I recorded that plant-parasitic nematodes were affected by the differences in the root glucosinolate profiles, whereas non-target organisms were not. The latter observation might be explained by the reduced intra-specific variation in the glucosinolate profiles of the root exudates compared to those of the roots. Even though total glucosinolate concentrations in roots and root exudates correlated positively, the number of individual glucosinolates that were recorded in the root exudates did not match those found in the roots. My experiments show that this may be due to different degradation rates of the individual glucosinolates in the soil.

By adding different soil communities to sterilized soils I examined whether belowground organisms can affect aboveground organisms via their shared host plant. For this, I used two cabbage cultivars that were highly divergent both in their effects on soil organisms and glucosinolate profiles. Microorganisms added to the soils promoted aphid population growth. The addition of nematodes tended to decrease aphid population growth. However, the effect of the soil organisms on aboveground organisms was similar for both cultivars, indicating that the outcome of below-aboveground interactions was not affected by intra-specific differences.

Genetic modification of plants could also indirectly affect plant growth, for example if the modification would affect the soil communities associated with the plants, which could subsequently affect aerial plant parts and aboveground processes. I explored this possibility by reviewing the recent literature on genetic modified plants, focusing on two case studies; rice plants modified to tolerate drought and salt stress and plants transformed to enhance their capacity to accumulate pollutants. Indeed, feedback loops between plants and the rhizosphere can result in both positive and negative feedback effects of the modified gene on aboveground plant properties. This may have unexpected consequences for the net effect of the genetic modification and eventually annihilate the positive effect of the modification on desired plant properties such as yield.

In this thesis I also showed that when evaluating genetically modified plants in greenhouse studies, effects on soil organisms are limited when compared to the field. I propose that this is due to the fact that greenhouse studies use relatively simple soils that lack the complex plant-soil interactions that can be present in the field. This is an indication that greenhouse studies have a limited predictive value for field effects on soil organisms. Greenhouse

studies are nevertheless useful for selecting a suitable and manageable set of varieties that are representative for the range of variation present in the full set of available cultivars. The selection of this sub-set can be made using the appropriate statistical tools, such as multivariate statistics. The selected sub-set can then be used as a baseline for more extensive studies assessing effects on (non-target) organisms in the field. I found that for root glucosinolate profiles this was a useful approach. Whether this holds true for other traits is yet to be assessed.

Before one can evaluate whether effects of genetically modified plants fall within or outside the effects of conventional plants, a good knowledge of the range of effects that can be observed for conventional varieties is required. In this thesis, I have provided the basis for this knowledge for white cabbage, especially for belowground interactions and to some extent on belowground-aboveground interactions. Conventional varieties already differ in their effects on soil organisms and these effects can potentially result in altered below-aboveground interactions, as was simulated by the addition of specific organism groups to the soil. This range of effects can serve as a baseline to determine whether effects of genetically modified white cabbage plants fall inside or outside the range of effects that can be observed for these conventional varieties.

Chapter 1

Introduction

Within species there is a wide range of variation in traits (Darwin, 1859). This variation, termed intra-specific variation, occurs naturally in wild plant populations. During the agricultural revolution the intra-specific variation present in wild populations has been used to select plants for agricultural use. The process of breeding and artificial selection of plant species that were suitable for agricultural practices resulted in novel varieties, adding to the intra-specific variation present in these species. More recently, technological developments have led to novel varieties through genetic modification.

Intra-specific variation in plants is manifested in single or multiple traits and can be observed as differences in morphology, physiology, metabolic profiles, and gene expression patterns (Gregor, 1946; Gregor & Watson, 1954; Ramirez & Sinclair, 1975; Broekgaarden, Poelman *et al.*, 2007b). Both natural and artificially enhanced intra-specific variation in plants has the potential to influence an array of processes in ecosystems. Intra-specific variation in plant traits can cause differences in invasion potential, plant fitness, and affect organisms aboveground as well as belowground (Barney, Di Tommaso *et al.*, 2005; Crutsinger, Collins *et al.*, 2006; Crutsinger, Reynolds *et al.*, 2008; Wurst, Van Dam *et al.*, 2008; Poelman, van Dam *et al.*, 2009; Lankau, 2010; Kos, Kabouw *et al.*, 2011b).

Some of the ecological processes that are influenced by intra-specific variation are relatively well understood, whereas others are not. For instance, effects of plant intra-specific variation on plant-animal interactions aboveground are relatively well studied. Effects of intra-specific variation have been recorded to cascade up multiple trophic levels, indicating how strong the effect can be (Crutsinger *et al.*, 2006; Crutsinger *et al.*, 2008; Poelman *et al.*, 2009). The mechanisms and the effect strengths of variation in shoot traits on aboveground food webs are generally well understood. However, it has been less intensively studied to what extent intra-specific variation in plants, either due to natural or to artificial selection, can influence soil organisms. Plants do not only show intra-specific variation in aboveground traits, but also in belowground traits that can for instance affect diversity of soil organisms (Bever, Westover *et al.*, 1997; Wardle, 2002; van Leur, Raaijmakers *et al.*, 2008; Degenhardt, Hiltpold *et al.*, 2009) and their feedback effects on plant performance (Schweitzer, Bailey *et al.*, 2008).

With the advent of genetically modified plants effects of plant traits on biodiversity received renewed interest, specifically the difference in effects between a genetically modified plant and its parental variety. Genetic modification of a plant can be achieved by over-expressing or knocking out

existing genes or by introducing genes from other species (James, 2008; James, 2010). Genetic modification can create new varieties that have effects on soil organisms or processes. These effects may fall outside the range of effects of existing conventional varieties. However, to establish whether the effects caused by genetically modified plants fall within or outside of the range of effects caused by conventional varieties, one first needs to determine the effects of conventional varieties, the so-called baseline effect.

The main objective of this thesis will, therefore, be to use conventional crop varieties to evaluate intra-specific plant variation in effects on soil food webs. The span of effects found in conventional crops can then be used as a baseline to evaluate whether genetically modified plants would fall within or outside the range of effects caused by existing crop varieties.

Effect of plants on soil organisms

Plants can both negatively and positively affect soil organisms. These effects can be mediated by many processes, including litter deposition and root exudation (Walker, Bais *et al.*, 2003a; Crutsinger *et al.*, 2008). It is estimated that about 20% of the photosynthetically fixed carbon is allocated to root exudates (Walker *et al.*, 2003a). The exudation of plant primary metabolites (e.g. sugars or amino acids) can promote the development of soil communities by providing nutrients to microbes in the rhizosphere (Jones, Nguyen *et al.*, 2009; Lesuffleur & Cliquet, 2010). On the other hand, toxic secondary metabolites in the exudates can negatively influence members of the soil community (Scheidemann & Wetzel, 1997; Rumberger & Marschner, 2003; Bais, Weir *et al.*, 2006; Wurst, Wagenaar *et al.*, 2010b). In exudates of *Arabidopsis thaliana,* for instance, 289 secondary metabolites have been identified (Walker, Bais *et al.*, 2003b). Carbon resources, however, are not only supplied by exudates but also by decaying plant material. The decomposition of organic material into inorganic molecules is one of the most important functions in ecosystems and is performed by several groups of organisms, particularly microorganisms.

The quality of litter and the composition of the root exudates are dependent on the plant variety (Zeng, Mallik *et al.*, 2003; Flores, Saxena *et al.*, 2005; Crutsinger *et al.*, 2008; Li, Liu *et al.*, 2009). As soil organisms are strongly dependent on these plant nutrients. Intra-specific differences in the

quality of these plant-derived nutrients thus can affect soil organisms. Indeed, differences in the litter composition between plant varieties have been demonstrated to influence a variety of soil organisms (Clark, Prihoda *et al.*, 2006; Crutsinger *et al.*, 2008). Differences in the quantity of toxic metabolites in root exudates also contribute to differences in the abundance and/or the community composition of soil organisms (Bressan, Roncato *et al.*, 2009).

Plant-soil feedbacks

An increasing number of studies reveal how soil organisms are influenced by plants and how these changes in soil communities in turn influence plant performance (for reviews see Wardle, Bardgett *et al.*, 2004; Ehrenfeld, Ravit *et al.*, 2005; Bardgett & Wardle, 2010) This reciprocal interaction between plants and soil biota has been named plant-soil feedback (Bever *et al.*, 1997). Plants can be both negatively and positively affected by soil organisms. For instance, plant-pathogenic microorganisms can accumulate in the rhizosphere, negatively affecting plant performance whereas decomposers release nutrients which generally results in positive effects on plant performance (Wurst, Dugassa-Gobena *et al.*, 2004). Therefore, plant-soil feedbacks have previously been found to have positive as well as negative effects on plant performance (Gange & Brown, 1989; Bever *et al.*, 1997; Klironomos, 2002; Wardle *et al.*, 2004; Kardol, Bezemer *et al.*, 2006; van Grunsven, van der Putten *et al.*, 2007; van der Putten, Bardgett *et al.*, 2009; Jiang, Han *et al.*, 2010; Miki, Ushio *et al.*, 2010).

Both positive and negative plant-soil feedback has been shown to affect large scale ecosystem processes. Release from negative plant-soil feedback effects can enhance plant invasions (Klironomos, 2002; Reinhart, Packer *et al.*, 2003), whereas selective plant-soil feedback contributes to diversity in plant communities, as well as plant succession (Kardol *et al.*, 2006). Plant-soil feedback can be affected by plant intra-specific variation, both in strength and direction. Firstly, different plant varieties can affect soil organisms to different extents (Schweitzer *et al.*, 2008), possibly leading to stronger or weaker feedbacks. Secondly, different plant varieties can respond differently to identical soil communities, as was shown for two varieties of *Barbarea vulgaris* differing in their chemical composition (van Leur *et al.*,

2008). Consequently, the net effect of all plant-soil feedbacks strongly depends on the plant variety and the soil community in the plant's substrate.

Below-aboveground interactions

Recent studies have shown that soil communities can affect the plant, subsequently resulting in effects on aboveground communities that are associated with the plant (Wardle *et al.*, 2004; van der Putten *et al.*, 2009; Bardgett *et al.*, 2010). These interactions between belowground and aboveground organisms are highly complex and dependent on the environmental conditions, the soil community, the aboveground community, the plant species, and possibly the plant variety.

For instance, earthworms and mycorrhizal fungi together can enhance aphid development rate on *Plantago lanceolata*, whereas each of these soil biota individually resulted in slower aphid development (Wurst *et al.*, 2004). This was related to increased food quality for the aphids, given that earthworms mobilized nitrogen and mycorrhizae increased the phosphorus supply to the plants (Wurst *et al.*, 2004). Soil organisms can also change the plant's metabolism and hence metabolites involved in defense mechanisms (Hol, Macel *et al.*, 2004; Wurst, Langel *et al.*, 2006), which can subsequently result in effects on aboveground organisms. For example, in the presence of earthworms iridoid glucosides (a secondary metabolite with a defensive function) increased in *Plantago lanceolata* (Wurst *et al.*, 2004). Wireworm feeding belowground increased the terpenoid level in leaves of *Gossypium herbaceum* so that it was more resistant to *Spodoptera exigua,* an aboveground herbivore (Bezemer, Wagenaar *et al.*, 2003). Although these interactions are highly complex, some general patterns have been found. For instance, a meta-analysis by Kaplan *et al.* (2008) revealed that herbivory consistently affected metabolites in roots and shoots that have a defensive function against aboveground herbivores, especially those metabolites that are induced in a tissue specific manner. These studies demonstrate that belowground interactions can lead to increased defenses in aerial parts, which may be detrimental for aboveground organisms.

Intra-specific variation in plants can potentially influence these below-aboveground interactions. Similar to plant-soil feedback mechanisms, this influence can be exerted in two different ways. First, different plant varieties

exposed to identical belowground communities can mediate aboveground effects in variety-specific ways. For example, *Plantago lanceolata* varieties differing in their metabolic composition responded in a chemotype-specific way, by increasing or decreasing their primary and secondary metabolism when exposed to herbivory by wireworms (Wurst *et al.*, 2008). Secondly, intra-specific variation can lead to different soil communities (see above), which may subsequently result in a different net effect of plant-soil feedback and a different quality of aerial plant parts.

Intra-specific variation due to genetic modification

Genetic modifications are mainly intended to increase yields by making plants more resistant against pathogens or more competitive to weeds (James, 2008; James, 2010). However, transformations in plants may not only alter targeted processes but also processes for which they were not intended. This includes pleiotropic effects on traits or processes that affect organisms for which the modification was not intended.

Evidence pointing in this direction comes from the introduction of genes producing Bt-proteins, one of the most commonly studied transformations (James, 2010). In one study, Bt modification in corn resulted in higher lignin concentrations than in its non-transformed counterpart. As lignin is only decomposed by specialized groups of soil microorganisms, an increased lignin concentration could cause nutrients to be released over a longer time frame. This might subsequently reduce soil erosion, or the higher lignin concentrations might cause Bt-proteins to accumulate in the soil and hence produce a toxic effect (Saxena & Stotzky, 2001). Indeed, in a laboratory experiment it was shown that aerial parts of Bt-transformed maize needed longer to decompose because their lignin concentrations were higher (Flores *et al.*, 2005). Nonetheless the ecological relevance was not clear as culturable soil microorganisms did not seem to be affected by this change in lignin concentration. In another study, also with Bt-transformed maize, a lower lignin content was recorded and this resulted in a lower mortality of woodlice, but again abundances of culturable bacteria and fungi were not affected (Escher, Käch *et al.*, 2001). The effects of Bt transformation on lignin content in these two maize studies were thus contradictory, which is surprising as they both used Bt-corn expressing Cry1AB (Escher *et al.*, 2001; Saxena *et al.*,

2001). Such contradicting results indicate that effects of introducing a modification can be context dependent; the same transformation in the same species can result in inconsistent effects on soil organisms. There may be many factors that may explain this inconsistency: the methods used to introduce the gene, the position of the inserted gene in the genome, the parental cultivar in which the gene is introduced, and soil characteristics. This indicates that in order to predict the effects of introducing a genetic modification on soil organisms we must evaluate the effects in multiple backgrounds and environments.

In addition to the "simple" effects of genetically modified plants on soil organisms, there might be more complex ecological effects due to the introduction of the gene in these plants. It is unknown whether possible effects on soil organisms also affect plant soil feedback mechanisms, as has been recorded in both natural systems and with conventional crops. Also, the consequences of introducing novel genes on above-belowground interactions of genetically modified plants have not yet been evaluated.

Objectives

The main objective of this thesis is to use conventional crops to evaluate how intra-specific variation in plant traits can affect soil food web composition. This can be used as a baseline to evaluate whether genetically modified plants would fall within or outside this range of effects observed for existing crop varieties. More specifically, I will evaluate effects of intra-specific variation in metabolic profiles of white cabbage cultivars on soil organisms, the mechanisms behind these effects, and the potential consequences that such effects on soil organisms can have on below-aboveground interactions. First I will select plants that show a high degree of intra-specific variation in metabolic profiles. Then I will evaluate if this intra-specific variation results in different soil communities in field situations. Additionally, I will examine whether these differences in soil organism communities, caused by plant intra-specific variation, can also feed back to aboveground food web interactions. Lastly, by reviewing literature, I will examine how genetic modifications can influence aerial parts of the plant through altered rhizosphere interactions.

Study system & experimental approach

In this thesis I will take a combined approach of greenhouse and field studies to establish effects of variation in plant traits on soil organisms, the mechanisms behind these effects, and possible consequences for below-aboveground interactions. In the greenhouse I will screen a large number of varieties for their degree of intra-specific variation in their chemical composition. As genetically modified crops can exhibit variation in traits or effects that are similar, smaller, or larger than those of conventional crops it is necessary to select the most divergent conventional cultivars, if these are to represent a baseline. Additionally, with this subset of most diverging varieties, I will perform a field study. This approach allows the screening of a larger number of plants for intra-specific variation in the greenhouse while reducing the number of varieties that have to be field-tested for effects on soil organisms.

However, this approach can only succeed if the intra-specific variation recorded in the greenhouse is consistent with the intra-specific variation in the field. That is, the most divergent varieties in the greenhouse should still be the most divergent varieties in the field. After these field experiments I will return to the greenhouse to test if effects of these varieties on soil organisms that have been recorded in the field have consequences for below-aboveground interactions.

Model system

As model plants I use the family of the Brassicaceae. From this family I will use white cabbages (*Brassica oleracea* var. *capitata*), as these plants show a high degree of intra-specific variation in metabolic profiles and other traits. However, I will also examine other members from other genera of the Brassicaceae family in order to find general patterns. Cabbages have been cultivated for centuries (Sturtevant, 1887) and it is generally thought that cabbages originated from the Mediterranean area. The first references to white cabbage are already found in Roman times (Sturtevant, 1887; Zeven, 1996; the Elder, around 160 BC). Due to this long history of cultivation in several parts of the world white cabbages show a high degree of selected intra-specific variation (Broekgaarden *et al.*, 2007b; Poelman, Broekgaarden *et al.*, 2008a). Intra-specific variation in white cabbages has been recorded, at least for aboveground parts, in morphology, chemistry, and gene expression

(Broekgaarden, Poelman *et al.*, 2007a; Kos, Broekgaarden *et al.*, 2011a). In addition to the long history of cabbage cultivation novel varieties are still being developed. New genetically modified genotypes of cabbages have been developed recently. These genetically modified cabbages produce Bt-proteins, a modification that was introduced with the intention of making the plants more resistant against insect pests (Metz, Dixit *et al.*, 1995; Paul, Sharma *et al.*, 2005).

Selection of soil organisms to evaluate effects of intra-specific variation

I will select a subset of relevant representatives of all soil organisms and monitor these to evaluate effects of intra-specific variation in white cabbage on soil organisms. To select groups of soil organisms and to maximize the relevance of the selected organisms for evaluating effects of intra-specific variation in chemical composition, several criteria can be used; 1) the economic and 2) ecological significance of the organism, 3) the probability of exposure (in this thesis to intra-specific variation in *Brassicaceae*), 4) sensitivity of the organism, and 5) the ability to represent a larger group of organisms (Dutton, Romeis *et al.*, 2003; Scholte & Dicke, 2005). I used these criteria for selecting the following organisms: earthworms, micro-arthropods (Collembola and mites), nematodes, and microorganisms.

Earthworms are of paramount importance to soil ecosystems as they affect soil texture and decomposition of organic material, thereby stimulating plant development. Therefore earthworms are of great economic importance (Pimentel, Wilson *et al.*, 1997). Micro-arthropods, such as Collembola and mites, are beneficial to soil quality as they stimulate microbial activity and selectively graze on microorganisms (Dunger & Fiedler, 1997). Micro-arthropods are sensitive to changes in soil quality and hence they are used in ecotoxicological studies and in tests of environmental impacts of intra-specific variation (Haughton, Champion *et al.*, 2003), including intra-specific variation in *Brassicaceae* varieties differing in chemical composition (Jensen, Styrishave *et al.*, 2010). Nematodes are another valuable and sensitive indicator group (Bongers, 1990; Holterman, Rybarczyk *et al.*, 2008). They are relatively easy to sample and can be assigned to specific trophic groups (e.g. bacterial feeding or plant pathogenic) based on the morphology of their mouthparts (Brimecombe, Leij *et al.*, 2001). Studies of *Brassicaceae* species have shown that differences in their chemical composition result in differences in the abundances of their

associated nematodes (Potter, Davies *et al.*, 1998; Potter, Vanstone *et al.*, 2000). Microorganisms have a critical function in ecosystems as they make up a large percentage of the soil biomass and are important for mobilizing nutrients. Several studies have demonstrated that intra-specific variation in *Brassicaceae* can affect the composition of the microbial community due to differences in plant chemistry (Rumberger *et al.*, 2003; Bressan *et al.*, 2009).

Glucosinolates

Brassicaceae are chemically characteristic because they contain glucosinolates, a class of plant metabolites that have been extensively studied. Glucosinolates are a group of over 120 nitrogen and sulfur containing metabolites found almost exclusively in plants of the Brassicales (Mithen, 2001). Plants producing glucosinolates also contain the enzyme myrosinase, which hydrolyses glucosinolates to isothiocyanates, thiocyanates and nitriles. Glucosinolates and their breakdown products are thought to have direct defensive functions by deterring generalist or attracting specialist herbivores and attracting natural enemies of these herbivores aboveground (Mithen, 2001; Brown, Tokuhisa *et al.*, 2003; Hopkins, van Dam *et al.*, 2009). Belowground, glucosinolates and isothiocyanates can confer resistance against bacteria, fungi and nematodes (Potter *et al.*, 1998; Rumberger *et al.*, 2003). *Brassica* species are also used for biofumigation (Kirkegaard & Sarwar, 1998), a process in which plants containing high concentrations of glucosinolates are incorporated into the soil with the aim of reducing the number of plant pathogens (Angus, Gardner *et al.*, 1994; Kirkegaard *et al.*, 1998; Kirkegaard, Sarwar *et al.*, 2000; Gimsing & Kirkegaard, 2009). Higher glucosinolate concentrations in the plant result in better disease suppression (Sarwar, Kirkegaard *et al.*, 1998b). Not only does the total concentration of glucosinolates influence interactions with soil organisms, but also the amount of individual glucosinolates may influence these interactions (Brown & Morra, 1997). For instance, the presence of one specific glucosinolate, 2-phenylethyl glucosinolate, is associated with reduced susceptibility of *Brassica* species to nematode infestation (Potter *et al.*, 1998; Potter *et al.*, 2000). *Brassicaceae* plants may also benefit from low glucosinolate concentrations as mycorrhizal fungi can only colonize plants during developmental stages in which glucosinolate concentrations are low (Pongrac, Vogel-Mikuš *et al.*, 2008). Glucosinolates can also affect non-herbivore soil organisms at higher trophic

levels or organisms not directly associated to the roots (non-target organisms) such as detritivores. Effects on these non-target organisms have been shown in laboratory studies with purified glucosinolates using Collembola and microorganisms (Rumberger *et al.*, 2003; Jensen *et al.*, 2010). Again, as observed for plant pathogens and herbivores, not only the total concentrations but also the amount of individual glucosinolates (*i.e.* the profiles) can affect non-target organisms.

Glucosinolates are constitutively produced, and are differentially allocated between above-and belowground parts (Kaplan *et al.*, 2008; van Dam, Tytgat *et al.*, 2009). Additionally, glucosinolate biosynthesis can also be upregulated by both above- and belowground herbivory (Birch, Griffiths *et al.*, 1992; Wurst *et al.*, 2006; van Leur *et al.*, 2008; Lohmann, Scheu *et al.*, 2009). However, the extent of induction of glucosinolates depends on the plant tissue that is attacked and also the identity of the attacker. For example, glucosinolates in aerial parts can be affected by root herbivory, although different types of responses have been observed, including both increasing and decreasing total concentrations and changes in profiles (Birch *et al.*, 1992; Wurst *et al.*, 2006). Systemically induced changes in glucosinolates are able to mediate below-aboveground interactions (Soler, Bezemer *et al.*, 2007). Aboveground organisms can either be attracted (e.g., natural enemies perceiving the volatile breakdown products of the glucosinolates) or deterred (e.g., some herbivores) as a result of the changes in leaf glucosinolate composition caused by belowground organisms. Because of the importance of glucosinolates for soil organisms as well as below-aboveground interactions, species of *Brassicaceae* differing intra-specifically in glucosinolates are suitable model species for constructing a baseline.

Thesis outline

In **chapter 2** of this thesis I describe variation in glucosinolate concentrations and profiles in both roots and shoots among twelve white cabbage cultivars. In addition, I test whether cultivar-specific differences in glucosinolates observed in the greenhouse are consistent in the field. I do this by selecting a subset of four cultivars, which represent the range of intra-specific variation present in all cultivars, including the most divergent cultivars. These four cultivars will serve to assess baseline effects in this thesis.

This baseline variation in glucosinolates is used in **chapter 3** to evaluate the effects of glucosinolates on a range of soil organisms from different trophic levels, which differ in their degree of association with plant roots. I consider decomposers such as earthworms and Collembola, predators such as mites, plant pathogenic nematodes, and bacteria and fungi.

In **chapter 4** I use a set of different species and cultivars of *Brassicaceae* to study to what extent the glucosinolates that are produced by these plants can be retrieved from the rhizosphere. This experiment is also intended to reveal mechanisms underlying the effects of glucosinolates on soil organisms. If non-target organisms are not affected in chapter 3, one possible explanation could be that glucosinolates are not exuded. I not only study the quantity of the recovered glucosinolates but also whether the glucosinolate profiles in the roots correspond to those in the rhizosphere. Additionally, I examine the degradation rate of the glucosinolates and whether this degradation rate depends on the biosynthetic origin of the glucosinolates and the absence/presence of microorganisms.

In **chapter 5** I test whether intra-specific variation in plants can alter the outcome of below-aboveground interactions. Plant intra-specific variation is represented by two distinctly different cabbage cultivars that I also use in the previous chapters. Plants are grown on soils without any soil organisms (sterilized soils), sterilized soil to which nematodes are added, or sterilized soil with microorganisms. Aboveground I study the population development of aphids, their glucosinolate profiles, and the fitness of the parasitoids of these aphids.

In **chapter 6** I discuss how the use of genetically modified plants could affect aboveground plant traits and plant fitness via indirect feedback loops with the rhizosphere.

Chapter 7 is a synthesis of the results from all chapters. In addition to discussing my own results I will give recommendations for evaluating the effects of intra-specific variation, such as effects of genetic modification, on soil organisms. These recommendations include statistical advice; I will highlight the baseline approach taken in this study and how baseline effects can be evaluated using the proper statistics. Effects of genetically modified plant can fall within or outside this baseline and good statistical measures are needed to determine this. Additionally, I will include recommendations on how to experimentally assess effects of intra-specific variation when using a combined greenhouse and field approach.

Chapter 2

Intra-specific differences in root and shoot glucosinolate profiles among white cabbage (*Brassica oleracea* var *capitata*) cultivars

Patrick Kabouw, Arjen Biere, Wim H. van der Putten & Nicole M. van Dam

Abstract

Shoot glucosinolate profiles of Brassicaceae are known to vary within species, across environmental conditions and between developmental stages. Here we study whether root profiles follow the intra-specific, environmental and developmental variation observed for aerial parts in white cabbage cultivars. We also assess whether greenhouse studies can be used to predict shoot and root glucosinolate concentrations and profiles in the field. Root glucosinolate profiles showed significant intra-specific variation, however, this variation was unrelated to that in shoot profiles. One of the strongest determinants of the diversity in the root profiles was 2-phenylethyl glucosinolate (gluconasturtiin). Root profiles were generally comparable between greenhouse studies and field trials, whereas shoot profiles were highly plastic. We conclude that among white cabbage cultivars shoot glucosinolate profiles are not indicative of root profiles. We further conclude that greenhouse assessments of root glucosinolates can be reliable predictors of root glucosinolate profiles in the field due to their low plasticity.

Keywords; Induced Response, Glucosinolates, 2-Phenylethyl Glucosinolate, Aboveground-belowground, Plant defense, Brassica oleracea

Introduction

Brassicas are economically important crops which show high intra-specific variation in morphological and chemical traits (Hanson, Yang *et al.*, 2009). Glucosinolates (GS), a structurally diverse group of about 120 nitrogen and sulfur containing secondary metabolites, are widespread within the Brassicaceae. Previous studies have shown that concentrations and profiles of GS show considerable variation within species, and that they vary with environmental conditions and developmental stage (Potter *et al.*, 2000; Rosa & Rodrigues, 2001; Castro, Aires *et al.*, 2004; Poelman, Galiart *et al.*, 2008b; Hanson *et al.*, 2009). Variation in GS has been of interest to ecologists and nutritional chemists alike. Ecological studies have investigated the effects of variation in *Brassica oleracea* GS concentrations and profiles on aboveground plant-insect interactions. Both generalists and specialists can be influenced by GS (Gols, Raaijmakers *et al.*, 2007; Poelman *et al.*, 2008b). Some GS may have anti-carcinogenic effects (Lund, 2003; Moreno, Carvajal *et al.*, 2006) and nutritional studies have mainly focused on these GS in the economically important aerial parts during the harvestable phase of the plants. Root GS levels, although less studied in the context of human health for which they also can be used (Smetanska, Krumbein *et al.*, 2007), are important for resistance against soil pests and may be used for biofumigation.

From the plant's perspective, GS in roots and shoots share the same function, *i.e.* defense again harmful organisms. Moreover, there is evidence for transport of GS and their precursors through the phloem (Merritt, 1996; Brudenell, Griffiths *et al.*, 1999; Chen, Petersen *et al.*, 2001), suggesting that shoots and roots exchange GS. This implies that their levels and profiles could be correlated. However, the few studies that have addressed this correlation show that GS levels are on average 4.5 fold higher in roots than in shoots and that the composition of the GS is also significantly different between these plant parts (Rosa, 1997; Castro *et al.*, 2004). Records on linkages between root and shoot GS levels and profiles are scarce and mainly focus on differences in the patterns of induction within a single plant or cultivar (van Dam *et al.*, 2009).

Only a few studies have investigated whether variation in shoot and root GS profiles and concentrations between cultivars is consistent across different environments and developmental stages. In aerial parts GS concentrations depend on a variety of factors including temperature, time of

day, water content, and nutrient supply (Rosa, Heaney *et al.*, 1994; Rosa, 1997; Pereira, Rosa *et al.*, 2002; Gols *et al.*, 2007). Even under controlled greenhouse conditions, GS concentrations in leaves of *Brassica* species fluctuate when sown over a time period of several months, which was ascribed to abiotic seasonal changes (Gols *et al.*, 2007). Soil characteristics like pH influence GS concentrations in leaves of kale (Petersen, Chen *et al.*, 2002; Velasco, Cartea *et al.*, 2007; Gerendas, Breuning *et al.*, 2008; Pongrac *et al.*, 2008). In addition, the developmental stage of the plant also affects both the concentration and the profiles of the GS (Petersen *et al.*, 2002). Biotic interactions may alter GS profiles as well. Aboveground plant-animal interactions can cause upregulation of specific GS, depending on the tissues attacked and the identity of the herbivore (Kusnierczyk, Winge *et al.*, 2007; Textor & Gershenzon, 2009). In *B. oleracea,* herbivory by the generalist *Myzus persicae* upregulates indolic GS (Kim & Jander, 2007) whereas damage resulting from the specialist *Pieris rapae* also increases aliphatic GS (Agrawal & Kurashige, 2003). There is especially little information on root GS.

Studies of root GS have mainly focused on the effects of specific root GS on soil (micro)organisms (Schreiner & Koide, 1993; Kirkegaard *et al.*, 2000; Potter *et al.*, 2000). The best studied root GS is 2-phenylethyl GS, which on average accounts for 40% of the total GS concentration in *Brassica* species (Rosa, 1997; van Dam *et al.*, 2009). The 2-phenylethyl GS degradation product, 2-phenylethyl isothiocyanate, is highly toxic to a range of soil organisms, such as fungi and root-feeding nematodes (Potter *et al.*, 2000; van Dam *et al.*, 2009). Currently, plant breeders are attempting to increase the GS concentration and specifically 2-phenylethyl GS in *Brassica* roots (Gimsing *et al.*, 2009) to apply them as biofumigants. Because the effect of 2-phenylethyl GS is concentration dependent it is important to know how consistent root GS profiles are in response to variable conditions. As it is easier to sample shoot tissue over time, it would be most practical for the selection process if root concentrations and profiles could be predicted from shoot samples, preferably from greenhouse trails.

Our study has two main aims. First we assess intra-specific variation in shoot and root GS concentrations and profiles in twelve different white cabbage cultivars and analyze whether there is a correlation between root and shoot GS. Second, we examine the stability of the profiles across environments and developmental stages in a subset of the cultivars covering the range of variation present in all twelve cultivars. Our results will show how stable root

and shoot GS are under these different conditions and if field GS concentrations and profiles can be predicted from greenhouse trials.

Material and methods

Cultivar selection

Twelve white cabbage cultivars were selected to cover a wide range of variation in either GS or in resistances/susceptibility to insect pests. These cultivars (with seed source and if known the breeder in brackets) were Badger Shipper (Centre for Genetic Resources NL, University of Wisconsin US), Bartolo (breeder and source Bejo seeds, NL), Bewama (Plant Research International, NL), Castello (breeder and source Nickerson Zwaan, NL), Christmas Drumhead (Centre for Genetic Resources, NL), Domia (Horticulture Research International, UK), Galaxy (breeder and source Seminis, NL), Jersey Queen (Centre for Genetic Resources, NL), Langendijker Bewaar (Plant Research International, NL), Lennox (breeder and source Bejo seeds, NL), Rivera (breeder and source Bejo seeds, NL) and Stonehead (breeder and source Sakata, Japan). All varieties are hybrids or inbred lines.

Seeds were germinated for ten days in a growth cabinet in a plastic container with glass beads and demineralized water. The growth cabinet had a day:night period of 16:8 hours and a temperature of 25:15°C respectively. Ten seedlings were transferred to 1L pots filled with 1100 grams of river sand (particle size distribution: <125 µm 13%; 125–250 µm 83%; >250 µm 4%). The surface surrounding the seedlings was covered with aluminum foil to reduce evaporation. Pots were relocated to a greenhouse with the following conditions (day/night): 25/15°C, 16/8 h, and a minimum PAR of 225 µmoles m^{-2} s^{-1}. At regular intervals, the plants were fertilized with an increasing amount of half strength Hoagland nutrient solution with double the amount of KH_2PO_4. In addition the pots were weighed and supplemented with water to maintain a water content of 14% based on dry sand mass once a week. Plants were harvested 29 days after planting. Roots were rinsed with tap water, dried with filter paper and both roots and shoots were frozen at -20°C within five minutes of harvest.

Induction experiment

One of the important aromatic glucosinolates (2-phenylethyl GS) appeared to be absent from some of the cultivars including Galaxy (see results). To assess whether this cultivar only lacked 2-phenylehtyl GS constitutively, or that it was also unable to produce this GS after induction, we performed an additional experiment. We tested for induction this aromatic GS in Galaxy after treatment with jasmonic acid to the roots. We only tested for induction in the roots as shoots of Brassicaea generally produce negligable amounts of this GS (van Dam *et al.*, 2009). Seeds of Galaxy were germinated on glass beads for ten days and transferred to 1L pots filled with river sand and placed in a greenhouse under the same conditions as above. After 28 days from relocation, ten plants were treated with 2.4 µmoles (0.5 mg) jasmonic acid (Sigma, St. Louis, IL, USA) in 10 ml 0.1% Triton (Sigma) injected near the root-shoot interface in the sand. Ten control plants were treated with the same amount of HCl to control for pH (=4) effects. Seven days after treatment all plants were harvested and their roots were washed and frozen at -20°C as above.

Greenhouse study

Based on previous experiments and results obtained from this study the Lennox, Rivera, Christmas Drumhead and Badger Shipper cultivars were selected to cover a substantial range of observed GS variation (Poelman *et al.*, 2008a). Seeds were germinated on glass beads for ten days. Ten seedlings per cultivar were transferred to 2L pots filled with 2000 grams of a sieved (5 mm mesh) and mixed loamy, sandy mineral soil from Mossel (Planken Wambuis; 52.06° north 5.75° east, The Netherlands; N =0.13%, C=2.1%, C/N = 16.7; particle size distribution: <2 µm 3%; 2–63 µm 17%; >63 µm 80%) before relocation to the greenhouse. This soil was known to contain a variety of beneficial and harmful soil organisms (Wurst & van Beersum, 2009). Plants were supplied twice with nutrients (half strength Hoagland nutrient solution with double the amount of KH_2PO_4) and once a week the pots were calibrated to achieve a soil moisture content of 19% of dry soil weight. The greenhouse conditions were as above. Roots were collected 56 days after relocation, at which the plants had similar sizes as in the cultivar selection experiment, and washed with tap water before storage at -20°C.

Field experiment

The same four cultivars as above were used in a field trial. The trail started on April the 28th 2008 with two week old seedlings and consisted of 32 plots with 49 plants of one cultivar per plot (8 plots per cultivar). The location of the plots was randomized. The field was located near Wageningen; 51.95° north 5.64° east, The Netherlands, with the following characteristics N =0.14%, C =1.7%, C/N =12; particle size distribution: <2 µm 5%; 2–63 µm 79%; >63 µm 19% (clay soil). On September 5th, so 130 days after germination, leaf material was collected. The third-youngest leaves of four plants per plot were removed with a scalpel and flash-frozen in liquid nitrogen. The leaf samples were transported to the lab in a plastic bag on ice and stored at -20°C. On the 8th of September, 133 day old roots of an additional two plants per plot were harvested and transported to the laboratory on ice, where the roots were cleaned with tap water and frozen at -20°C. All samples were pooled per plot.

Glucosinolate analysis

Roots and shoots were freeze-dried and GS were extracted from finely ground plant material (100 mg) by boiling in 70% MeOH, washing 2x 1 ml 70% MeOH, 2x 1ml milliQ and 2ml NaOAC before being desulfatased for 16 hours with 20µm arylsulfatase which contained 24 units/g solid product (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 (also Sigma) column. The following days the eluate was freeze-dried, redissolved in 1ml milliQ and filtered over a 20µm filter. Separation of the GS was performed with a reversed phase C-18 column on a HPLC with an acetonitril/milliQ gradient. GS detection was performed with a Photodiode Array detector with 229 nm as the integration wavelength. We used the correction factors at 229 nm from Buchner (1987) the EC (1990) and Brown, Tokuhisa *et al* (2003) to calculate the concentrations of the GS. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and ultraviolet spectra with those of standards which were provided by M. Reichelt, MPI Chemical Ecology, Jena (Germany) and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). Different concentrations of 2-propenyl GS (ACROS, NJ, USA) were used as external standard and extracted following the same procedure as the plant material. GS levels were calculated on the basis of dry weight.

Statistical analysis

Student's *t*-tests were used to analyze differences in GS levels between roots and shoots, and between the two time points in the field trial. Pearson correlations were used to analyze associations between root and shoot GS concentrations. Significance values were corrected with a Bonferroni correction ($\alpha' = \alpha/n$) to adjust for multiple testing. Differences in GS between cultivars were studied using analysis of variance (ANOVA). When ANOVA assumptions could not be met, a non-parametic Kruskal-Wallis test was used. For all these analyses GS were grouped on the basis on their biosynthetic origin (Aliphatic, Indole, Aromatic, and Total). Values were transformed, if needed, to achieve normality and homogeneity of variances. Tests were performed in Statistica 8.0 (StatSoft, Tulsa, OK, USA).

Ordination diagrams in CANOCO (Biometris, Wageningen, NL) were used to visualize the variation in profiles within and between roots and shoots. Detrended correspondence analysis was used to check the length of the gradient. Depending on the score either principal component analysis (PCA) or correspondence analysis (CA) was used for visualization. The former appeared to be the most appropriate method for shoots, the latter for roots and these were used accordingly. If needed data was log transformed. Cultivar differences in both the ordination diagrams were examined by taking the sample scores of the ordination diagrams. Thereby we focused on inter-sample distances on the significant axes (determined by Scree plots of root CA diagrams and parallel analysis with Monte Carlo permutation tests for shoot PCA diagrams) and checking the significance with an ANOSIM on the basis of Euclidean distances in the program PAST (Version 1.89) (Hammer, Harper *et al.*, 2001). Significance values were corrected with a Bonferroni correction. The overall significance was checked with a PCA by checking the significance between the cultivars on the first and all other axes by Monte Carlo permutation test in the program CANOCO. The similarity of the clustering between the root in the CA plot and shoot in the PCA plot was checked by examining the correlation between the two by a Mantel test. For this test the Euclidean distances between the cultivars that were generated by the ANOSIM permutation test were compared to each other.

Results

Differences in GS between roots and shoots

Both roots and shoots of the twelve cultivars contained aliphatic, indole and aromatic GS (**Table 2.1**). Root concentrations were on average 3.6 fold higher than shoot concentrations (average root concentration 11.5 µmol/g ± 4.8 SD, average shoot concentration 3.1 µmol/g ± 1.7 SD, Student's *t*-test, P< 0.001). There was no significant correlation between root and shoot concentrations of total (P= 0.29, R^2= 0.01), aliphatic (P= 0.12, R^2= 0.03) or indole GS (P= 0.14, R^2= 0.02). When studied at the level of individual GS, only two GS showed significant correlations between shoot and root concentrations: the aliphatic 2-propenyl GS (P< 0.001 R^2= 0.49) and 2-phenylethyl GS, the only aromatic GS recorded in this study (P= 0.001, R^2= 0.25).

Table 2.1: Glucosinolates recorded in root and shoots in the cultivar selection experiment, with abbreviations and trivial name, GS are grouped based on their biosynthetic origin. The number indicates in how many of the cultivars the glucosinolate was recorded.

Abbreviation	Trivial name	Scientific name	root	shoot
Aliphatic				
IBE	glucoiberin	3-methylsulphinylpropyl	12	12
SIN	sinigrin	2-propenyl	12	12
RAPH	glucoraphanin	4-methylsulphinylbutyl	12	12
IBV	Glucoiberverin	3-methylthiopropyl	12	12
ERU	Glucoerucin	4-methylthiobutyl	12	9
PRO	progoitrin	2-OH-3-butenyl	12	12
EPRO	epiprogoitrin	2-(*S*)-2-Hydroxy-3-butenyl	9	7
GNA	gluconapin	3-butenyl	11	12
Indolyl				
4OH	4-hydroxyglucobrassicin	4-OH-3-indolylmethyl	12	12
4MeOH	4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl	12	11
NEO	neo-glucobrassicin	1-methoxy-3-indolylmethyl	12	12
GBC	glucobrassicin	3-indolylmethyl	12	12
Aromatic				
NAS	gluconasturtiin	2-phenylethyl	10	8

Variation in the GS profiles was larger in the roots than in the shoots, as visualized in **Figure 2.1**; root samples cluster further apart than shoots samples. The GS profiles of the cultivars showed no significant correlation between root and shoots (Mantel test comparing the positions in both ordination diagrams P>0.1). The most dominant GS in both shoots and roots was 2-propenyl GS, which made up 33.2% of the total GS concentration in shoots and 18.0% in roots. In shoots, the second most dominant GS was 3-methylsulphinylpropyl (29.1%), the only GS that was found in higher concentrations in shoots than in roots (**Figure 2.1**). In roots the second most dominant GS was 3-methylthiopropyl GS (16.0%) followed by 4-methoxy-3-indolylmethyl GS (10.1%) and 2-phenylethyl GS (9.2%). In the shoots 2-phenylethyl GS was only recorded in trace amounts (**Table 2.2**).

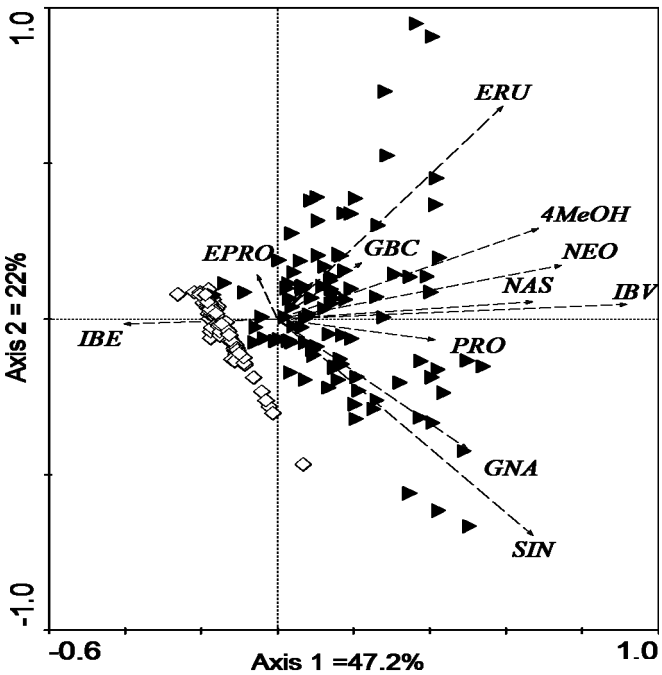

Figure 2.1 PCA plot of root and shoot glucosinolate profiles of 12 cabbage cultivars (for abbreviations see Table 2.1). ▶ Represent root samples ◇ represent shoot samples.

Differences in GS between cultivars

Shoot GS concentrations (total, indolyl, aromatic and aliphatic) showed significant cultivar differences. In roots both the aromatic and aliphatic GS concentrations differed significantly between cultivars, whereas the indolyl and total GS levels did not (**Table 2.2**).

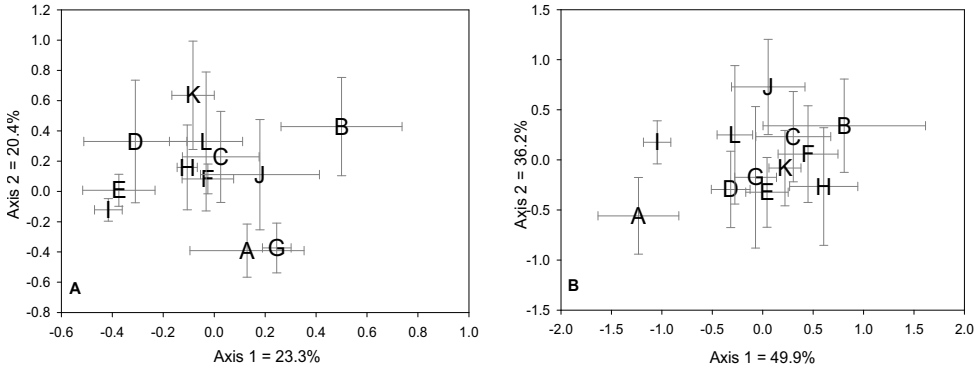

Figure 2.2 **A**) CA ordination diagram of the first two axes of root glucosinolate profiles of the 12 cultivars based on inter-sample distances the positions of the points are the mean positions of ten replicas and the associated standard deviation. Letters indicate the cultivars (A=Castello, B=Badger Shipper, C= Christmas Drumhead, D=Domia, E=Bewama, F=Rivera, G=Galaxy, H=Bartolo, I=Stonehead, J=Jersey Queen, K=Lennox, L=Langendijker Bewaar). **B**) PCA ordination diagram of the 2 significant axes of shoot glucosinolate profiles of the 12 cultivars the based on inter-sample distances.

To examine differences in root GS profiles between cultivars, the root GS were plotted in a CA diagram (**Figure 2.2a**).Euclidean distances between the cultivars were calculated by taking the sample loadings on the first four axes, which explained 75.4 percent of the variation. The R values of this analysis, obtained by ANOSIM permutation tests, are shown in **Table 2.3** with significant values highlighted. This demonstrates that root GS profiles differ significantly among cultivars even though the total concentration does not. 2-Phenylethyl GS, the only aromatic GS found in this study, makes a major contribution to these significant differences; it had the best fit to the first and third axes and the second best fit to the second and fourth axes in the PCA plot. This GS was totally absent from the cultivars Jersey Queen (J in **Figure 2.2a**) and Galaxy (G). Even though treatment of Galaxy plants with jasmonic acid significantly increased the total GS levels in their roots (from 7.5 µmol/g ± 2.0 SD in the control plants to 11.7 µmol/g ± 2.1 SD in the jasmonic acid treated plant, Student's *t*-test, P< 0.001), 2-phenylethyl GS was still not detectable in the roots of these treated plants. , Student's *t*-test, P< 0.001), 2-phenylethyl GS was still not detectable in the roots of these treated plants.

Table 2.2: Glucosinolate concentration from the greenhouse cultivar selection experiment in µmol/g dry weight (means ± standard error)

Cultivar	Aliphatic root	Indole root	Aromatic root	Aliphatic shoot	Indole shoot	Aromatic shoot	Total root	Total shoot
Badger Shipper	9,76 ± 1,31 a,b*	4,05 ± 0,10 a	0,05 ± 0,03 a	3,11 ± 0,67 a,b	0,76 ± 0,11 a	0 ± 0 a	13,85 ± 1,84 a	3,86 ± 0,74 a
Bartono	8,13 ± 0,96 a,b	2,74 ± 0,21 a	2,74 ± 0,37 b	3,43 ± 0,61 a,b	0,46 ± 0,08 a,b	0,02 ± 0,01 a,b	13,60 ± 1,32 a	3,91 ± 0,68 a
Bewama	7,71 ±1,13 a,b	2,60 ± 0,54 a	3,17 ± 0,61 b	2,78 ± 0,27 a,b	0,65 ± 0,10 a,b	0,04 ± 0,01 a,b	13,48 ± 2,09 a	3,47 ± 0,34 a
Castello	10,36 ± 1,57 a	2,64 ± 0,30 a	0,09 ± 0,09 a	3,80 ± 0,53 a	0,59 ± 0,10 a,b	0 ± 0 a	13,09 ± 1,80 a	4,39 ± 0,60 a
Christmas Drumhead	6,47 ± 0,87 a,b	5,55 ± 1,74 a	2,04 ± 0,28 b	2,04 ± 0,40 a,b,c	1,29 ± 0,41 a	0,01 ± 0 a,b	14,06 ± 1,96 a	3,33 ± 0,79 a,b
Domia	4,74 ± 0,69 a,b	3,26 ± 0,54 a	2,01 ± 0,19 b	2,70 ± 0,26 a,b	0,68 ± 0,09 a,b	0,04 ± 0 b	10,01 ± 1,08 a	3,41 ± 0,32 a,b
Galaxy	7,91 ± 1,00 a,b	3,61 ± 0,86 a	0 ± 0 a	2,75 ± 0,55 a,b,c	0,50 ± 0,09 a,b	0 ± 0 a	11,52 ± 1,36 a	3,25 ± 0,63 a,b
Jersey Queen	4,89 ± 0,65 a,b	3,12 ± 0,31 a	0 ± 0 a	1,10 ± 0,28 c	0,46 ±0,16 a,b	0 ± 0 a	8,00 ± 1,63 a	1,56 ± 0,40 b
Langendijker Bewaar	4,35 ± 0,66 b	3,37 ± 0,32 a	0,41 ± 0,14 a,b	1,79 ± 0,35 b,c	0,51 ± 0,15 a,b	0,01 ± 0,01 a,b	8,13 ± 0,95 a	2,31 ± 0,46 a,b
Lennox	4,65 + 1,06 b	0,43 ± 0,05 a	1,17 ± 0,27 a,b	2,47 ± 0,27 a,b,c	0,43 ± 0,05 a,b	0,01 ± 0 a,b	8,07 ± 1,63 a	2,90 ± 0,30 a,b
Rivera	6,73 ± 0,75 a,b	3,03 ± 0,32 a	1,75 ± 0,33 b	2,38 ± 0,38 a,b,c	0,59 ± 0,11 a,b	0,02 ± 0,01 a,b	11,50 ± 1,24 a	2,99 ± 0,46 a,b
Stonehead	4,61 ± 0,60 a,b	2,64 ± 0,41 a	1,25 ± 0,14 a,b	2,03 ± 0,18 a,b,c	0,21 ± 0,04 b	0,03 ± 0,02 a,b	8,50 ± 1,02 a	2,27 ± 0,22 a,b

Table 2.3: R values obtained with ANOSIM permutation test on the intersample distances of (1) the first 4 CA axes for the roots (values below the diagonal), explaining 75.4% of the variation, and (2) the first 2 PCA axes for the shoots (values above the diagonal), explaining 86.1% of the variation; * indicate significant dif erences.

	Castello	Badger Shipper	Christmas Drumhead	Domia	Bewama	Rivera	Galaxy	Bartono	Stonehead	Jerry Queen	Lennox	Langedijker bewaar
Castello	-----	0.6651*	0.8236*	0.6106*	0.7815*	0.8377*	0.6099*	0.7596*	0.4118	0.8568*	0.8455*	0.6298*
Badger Shipper	0.6778*	-----	0.0619	0.5131*	0.3667*	0.0004	0.1647	0.0827	0.5606*	0.0883	0.1642	0.2376
Christmas Drumhead	0.6389*	0.4020*	-----	0.6697*	0.4390	0.0022	0.0645	0.1411	0.9184	0.0833	0.1714	0.1957
Domia	0.4627*	0.4369*	0.2104	-----	0.1669	0.5767*	0.1153	0.5224*	0.5615*	0.6927*	0.5164*	0.1033
Bewama	0.6302*	0.7009*	0.5584*	0.2316*	-----	0.3378	0.0762	0.2818	0.8593*	0.5920*	0.0764	0.2518
Rivera	0.7276*	0.6380*	0.4458*	0.3838*	0.6100*	-----	0.1900	0.0082	0.7260*	0.2422	0.0671	0.3404*
Galaxy	0.0402	0.6669*	0.6516*	0.5236*	0.7353*	0.7484*	-----	0.2258	0.4346*	0.2014	0.1613	0.01933
Bartono	0.6398*	0.5258*	0.2602*	0.2573*	0.5040*	0.2231	0.6684*	-----	0.5970*	0.3649*	0.0804	0.3953*
Stonehead	0.7867*	0.8076*	0.7020*	0.2016*	0.5111*	0.9304*	0.8351*	0.7478*	-----	0.8652*	0.9260*	0.3918
Jerry Queen	0.2922*	0.1804	0.1611	0.1838	0.4471*	0.4276*	0.2498*	0.3669*	0.5587*	-----	0.4219	0.1569
Lennox	0.5893*	0.3540	0.3527	0.1324	0.5031*	0.3798*	0.6073*	0.3551*	0.5733*	0.2393	-----	0.3753*
Langedijker bewaar	0.3573*	0.3153	0.2584	0.0673	0.4102*	0.4169*	0.3324*	0.3102*	0.3851*	0.0926	0.0027	-----

Shoot GS profiles were plotted separately in a PCA plot (**Figure 2.2b**). The first two axes explained 86.1 percent of the variation, whereas the remaining axes did not significantly explain variation when examined by Monte Carlo Parallel analysis. R values between the cultivars are listed in **Table 2.3**. Castello and Stonehead (A and I, respectively, in **Figure 2.2b**) had relatively high 3-butenyl (2.26 and 1.27 μmol/g) and 2-propenyl (72.4 and 81.3 μmol/g) GS concentrations compared with the other 10 cultivars (mean (SE = 0.50 ± 0.14 and 28.9 ± 4.3 μmol/g,respectively).

Variation in GS profiles across environments and due to developmental stage

The four cultivars that were used to represent cultivar GS variation were grown in three different environments and to different developmental stages to examine the consistency of their GLS profiles. Since the plants not only experienced different environments but were also harvested at different ages during vegetative development (see Materials and Methods), consistency of GS patterns would indicate that GS patterns are not only conserved in the face of (a-)biotic environmental variation but also across different time points during vegetative development. The root GS profiles of these cultivars were plotted in a PCA diagram with the first axis fixed to plot the different environments and the second axis explaining 33.9% of the variation (**Figure 2.3**). The differences in root GS profiles between the cultivars grown in the three different conditions were largely consistent between the greenhouse and field experiments even though they were harvested at different time points after the start of the experiment. Badger Shipper consistently clustered apart from the other three cultivars due to its low 2-phenylethyl concentration. The other three cultivars remained closer together. This consistency of profiles is remarkable considering the variation in absolute concentrations among the cultivars (in both greenhouse studies GS concentrations significantly differed from those in the field study, **Table 2.4**, Kruskal-Wallis test $P<0.001$).

GS concentrations in the shoots of the four cultivars were significantly lower in the greenhouse (6.8± 4.6 SD μmol/g) than in the field (15.6± 7.3 SD μmol/g, Student's *t*-test, $P< 0.001$). In addition, profiles in the field profiles were more diverged than in the greenhouse even though in the field we pooled samples from four different plants. Hence the variation recorded is likely to be an underestimation of the individual plant variation present in the field (**Figure 2.4**).

Table 2.4: Root glucosinolate in µmol/g dry weight concentrations over the different environments (means of all samples per cultivar ± standard error) numbers indicate the ranging between the cultivars

	Cultivar selection experiment	Greenhouse study	Field study
Badger shipper	13.85 ± 1.84[2]	13.73 ± 0.84[1]	17.59 ± 3.16[2]
Christmas Drumhead	14.06 ± 1.96[1]	7.09 ± 0.76[4]	13.66 ± 2.65[4]
Rivera	11.50 ± 1.24[3]	7.26 ± 0.86[3]	18.29 ± 2.46[1]
Lennox	8.07 ± 1.63[4]	10.36 ± 0.63[2]	16.73 ± 4.53[3]

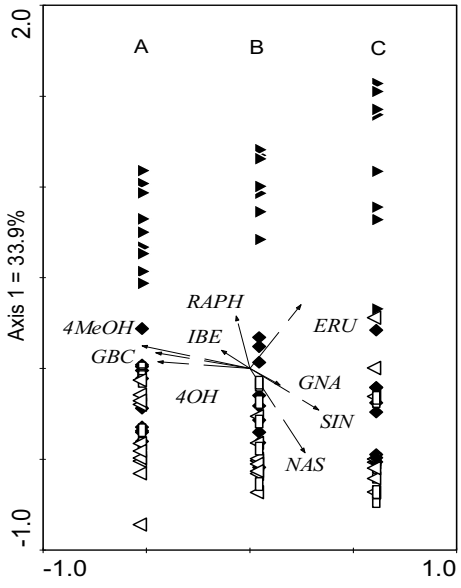

Figure 2.3 PCA plot of root glucosinolate profiles from the four selected cultivars. First (A) Cultivar selection experiment on a sandy soil, (B) greenhouse study on mixed loamy, sandy mineral soil (C) field study. □ Rivera, ◁ Lennox, ◆Christmas drumhead, ▶ Badger Shipper . For abbreviations of the GS see Table 2.1.

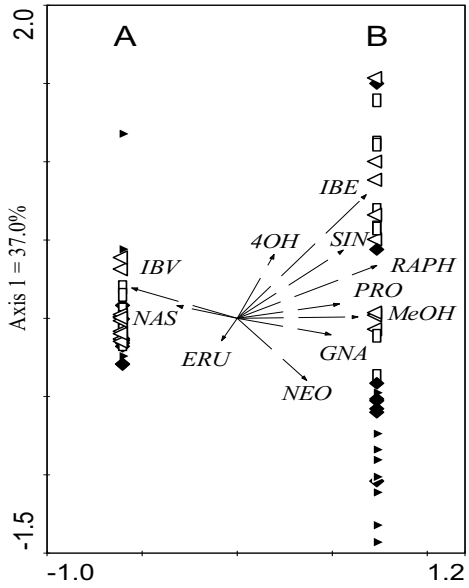

Figure 2.4 PCA plot of shoot glucosinolate profiles from the four selected cultivars. (A) Cultivar selection experiment on a sandy soil (B) field study. □ Rivera, ◁ Lennox, ◆Christmas drumhead, ▶ Badger Shipper. For abbreviations of the GS see Table 2.1.

Discussion

Here we demonstrate that profiles and total concentrations of GS in shoots and roots of white cabbage cultivars were not significantly correlated. We recorded that GS concentrations are circa three and a half-fold higher in roots than in shoots. The differences in concentrations are not due to a specific GS but more due to overall higher GS levels in roots. This seems to be a general pattern, except at early stages of development and during flowering, when GS concentrations belowground can be lower than or equal to those in aerial parts (Kirkegaard *et al.*, 1998; van Dam *et al.*, 2009). Here we did not analyze the concentrations during flowering or during the seedling phase as this is less relevant for white cabbage, which is normally harvested well before flowering. It should be noted that GS concentrations are commonly expressed on a tissue dry weight basis, as in our study. However, since the water content can vary between roots and shoots, the relative difference between shoot and root concentrations may therefore be different when expressed at a tissue fresh weight basis. Nevertheless, given the large magnitude of the reported difference, expression of concentrations on a different basis is not likely to qualitatively alter the conclusion that roots have higher GS concentrations than shoots which is also consistent with previous studies (van Dam *et al.*, 2009).

The independence of root and shoot GS profiles could be of value to agricultural practice if it would mean that we can independently select for root and shoot GS. On the other hand, the lack of correlation between root and shoot profiles is rather unfortunate as it means that measurements of shoot GS have a limited predictive power for the less easy to measure root GS profiles. The underlying causes of the differences between shoot and root GS profiles are unclear. It has been argued that differences in root and shoot GS concentrations and profiles in wild Brassica species might be due to differential selection pressures on glucosinolates in the above- and belowground compartment. For instance, in the soil the breakdown products of the GS might persist longer than in the air.

Therefore aerial parts might invest in GS that results in short lived volatiles whereas shoots can invest in GS that result in longer lived breakdown products. The breakdown products of 2-phenylethyl GS, the main GS responsible for the diverging profiles of root and shoot, are relatively hydrophobic, which makes them less likely to be leached from the soil, and are

also relatively non-volatile which also reduces loss from the soil (Sarwar *et al.*, 1998b; Laegdsmand, Gimsing *et al.*, 2007). Secondly, there might also be a physiologic basis for the observed differences in root and shoot GS, the most plausible one that both organs have different regulation mechanisms for the GS biosynthesis (van Dam *et al.*, 2009). Several GS transcription factors have been proved to be highly compartmentalized resulting is tissue specific expression (Gigolashvili, Yatusevich *et al.*, 2009). Whatever the underlying causes of the differences in root and shoot GS profiles are, it means that shoot GS profiles have limited predictive power for root profiles and vice versa.

Three of the twelve cultivars contained little or no 2-phenylethyl GS. Jasmonic acid induction could not alter this in the cultivar Galaxy, which indicates that it is not only constitutively absent, but that it can also not be induced by mimicking herbivore damage. This observation is in contrast with previous studies on other Brassicaceous species. In those studies, root concentrations of aromatic GS and specifically 2-phenylethyl GS, were generally higher than those recorded here and in some cases could be increased by jasmonic acid application (Ludwig-Müller, Schubert *et al.*, 1997; van Leur, Raaijmakers *et al.*, 2006). The fact that we found variation among our cabbage cultivars indicates that is difficult to extract general patterns from analyzing single species or cultivars. The intraspecific variation of 2-phenylethyl GS we recorded may influence soil organisms, such as fungi and nematodes (Potter *et al.*, 2000). For example, it has been hypothesized that the non-mycorrhizal status of *Brassica* species might at least in part be due to overall high levels of 2-phenylethyl (Olivier, Vaughn *et al.*, 1999; Vierheilig, Bennett *et al.*, 2000). Our results suggest that white cabbage cultivars with their high intra-specific variation in this GS are a good model system to investigate effects of 2-phenylethyl GS on root pests and other soil organisms.

Roots and shoots also differed in their responsiveness to environmental conditions and developmental stage. Shoot GS profiles were highly plastic and showed considerable differences between field and greenhouse-grown plants. This plasticity could reflect ontogenetic changes during vegetative development as well as responses to different (a)biotic conditions in the environment. Root profiles, on the other hand, were much more constant across different experiments. This was contrary to our expectation that root GS profiles would substantially differ between de greenhouse studies conducted on the loamy soil, that is rich in pathogenic soil organisms (Wurst *et al.*, 2009), as compared to the more sterile sandy soil as a result of differential induction. Also the presumed higher variability in

(a)biotic conditions in the field as compared to the greenhouse did not result in more variable root GS profiles in the field. As is postulated by optimal defense theory the different patterns between roots and shoots may be a reflection of differences in their defensive strategy (Karban, Agrawal *et al.*, 1999a; van Dam *et al.*, 2009). Although both roots and shoots are continuously challenged by herbivores and pathogens, it has been speculated that roots might be more constantly exposed to herbivory and therefore require a constantly high level of defense expression (Karban, Agrawal *et al.*, 1999b), whereas shoots may experience more variable biotic stresses, thus making it profitable to respond by inducible defenses, instead of allocating limited resources to constitutive defenses (van Dam *et al.*, 2009).

Our study does not allow us to disentangle effects of development stage (age at harvest) and effects of abiotic or biotic factors on GS profiles, nor was it intended to do so. But whatever the underlying causes of observed GS variation in our experiments were, we can conclude that GSs in aerial parts have limited predictive value for root GS profiles or their concentrations. Interestingly, root profiles of plants in the field could be more reliably predicted from greenhouse studies than shoot profiles, because root profiles were less plastic under different environmental conditions or were less dependent on development stage. This enhances the opportunities for ecologists or agronomists interested in the effects of specific GSs on soil ecosystems to extrapolate the results of greenhouse studies to the field. In contrast, this cannot be said for shoot GSs because we found these to be highly plastic.

Chapter 3

Effects of intraspecific variation in white cabbage (*Brassica oleracea* var. *capitata*) on soil organisms

Patrick Kabouw, Wim H. van der Putten, Nicole M. van Dam, and Arjen Biere

Abstract

Intraspecific variation in plants can affect soil organisms. However, little is known about whether the magnitude of the effect depends on the degree of interaction with the roots. We analyzed effects of plant intraspecific variation on root herbivores and other soil organisms that interact directly with living plant roots, as well as on decomposer organisms that interact more indirectly with roots. We used four different white cabbage (*Brassica oleracea* var. *capitata*) cultivars exhibiting a high degree of intraspecific variation in root glucosinolate profiles. Intraspecific variation affected root-feeding nematodes, whereas decomposer organisms such as earthworms and Collembola were not affected. Root-feeding nematodes were most abundant in one of the cultivars, Badger Shipper, which lacked the glucosinolate gluconasturtiin. The effect of the intraspecific variation in glucosinolate composition may have been restricted to root-feeding nematodes due to the rapid degradation of glucosinolates and their breakdown products in the soil. Additionally, the low biomass of root-feeding nematodes, relative to other soil organisms, limits the possibility to affect higher trophic level organisms. Our results show that variation in root chemistry predominantly affects belowground herbivores and that these effects do not extend into the soil food web.

Keywords; Glucosinolates, Trophic level, 2-Phenylethyl, *Pratylenchus*

Introduction

Soil food webs include a wide variety of organisms feeding on a broad range of different resources. Their basal resources are of plant origin, such as living roots, recalcitrant and labile plant litter, and root exudates. Plant roots are important factors in determining the composition of soil food webs (Witt & Setälä, 2010). Depending on the trophic level in the soil food web, the level of interaction with the plant differs. Root feeders directly interact with living plant roots, whereas detritivores and predacious soil organisms feed on plant litter or on second and higher trophic level organisms (de Ruiter, Neutel *et al.*, 1995; Mulder, Zwart *et al.*, 2003; Berg & Bengtsson, 2007; Holtkamp, Kardol *et al.*, 2008; Mulder & Lotz, 2009), leading to a more indirect interaction with living plant roots (Wardle *et al.*, 2004). Due to their direct exposure to the roots, root-associated and lower trophic level organisms in the soil food web are more likely to be affected by variation in plant quality than other soil organisms (Wardle, 2006). Nevertheless, decomposer organisms and higher trophic level organisms can potentially be influenced via changes in the quality and quantity of litter, in root-exudates, or via differences in the quality or quantity of prey or host species (Mulder *et al.*, 2009). In the present study, we examine how intra-specific variation among plant cultivars may affect these different components of the soil food web.

The effects of intraspecific plant variation have been well studied for aboveground food webs (Crutsinger *et al.*, 2006; Broekgaarden *et al.*, 2007b; Poelman *et al.*, 2009). Soil organisms could also be affected by intraspecific variation in plants, for example via chemical variation between plant roots, e.g. differences in the production of toxic compounds that affect soil organisms. However, belowground effects of intraspecific plant variation on plant-animal interactions have only been studied to a limited extent and mostly within single organism groups (Griffiths, Caul *et al.*, 2005; Wardle, 2006; Andersen, Sausse *et al.*, 2007; Wurst *et al.*, 2008).

To examine the effect of intraspecific variation we used a model system consisting of White Cabbage (*Brassica oleracea* var. *capitata*) cultivars which exhibit a high degree of intraspecific variation, both aboveground and belowground (Broekgaarden *et al.*, 2007b; Poelman *et al.*, 2009). The roots of these cabbage cultivars are known to differ in their glucosinolate composition (chapter 2). Glucosinolates are a group of secondary plant compounds prominently present in the Brassicaceae which could potentially influence the

soil food web (Yang, Zhu *et al.*, 2009). Upon tissue damage, glucosinolates are degraded by the enzyme myrosinase, thereby forming toxic breakdown products such as (iso)thiocyanates (Wang, Rosen *et al.*, 2009). Belowground, glucosinolates and their breakdown products are known to reduce the abundance of phytophagous organisms such as root-feeding nematodes (Potter *et al.*, 1998; Potter *et al.*, 2000; Lazzeri, Curto *et al.*, 2004), fungi (Rumberger *et al.*, 2003; Snapp, Date *et al.*, 2007; Bressan *et al.*, 2009), and bacteria (Rumberger *et al.*, 2003; Aires, Mota *et al.*, 2009; Bressan *et al.*, 2009). Aromatic glucosinolates, particularly gluconasturtiin and its breakdown product 2-phenylethyl isothiocyanate, are generally considered as the most toxic glucosinolates in plant roots (Potter *et al.*, 1998; Potter *et al.*, 2000; Vierheilig *et al.*, 2000; Rumberger *et al.*, 2003; van Dam *et al.*, 2009).

Besides exerting negative effects on root feeding organisms, glucosinolates can potentially influence decomposer organisms and higher trophic levels in the soil food web. Indeed, it has been shown in laboratory experiments that glucosinolates and their hydrolysis products affect Collembola (Jensen *et al.*, 2010) and entomopathogenic nematodes (Ramirez, Henderson *et al.*, 2009), although these taxa are not directly feeding on living plant roots.

The objective of the present study is to examine the effects of intraspecific variation among plants on components of the soil food web which differ in their degree of exposure to variation in plant chemistry. Therefore we analyzed the fate of organisms that feed on living plant roots and organisms belonging to the detritus-based food web. These soil organisms differ in their nutrient requirements, body size, and growth/reproductive rates (Mulder *et al.*, 2009). We tested the hypothesis that the intraspecific chemical variation in our plants will affect root-feeding nematodes due to their close contact with the roots and we determined the extent to which this variation influences other important groups such as mites, Collembola, earthworms, and enchytraeids in the soil food web.

Material and methods

Cultivar and field experiment

To test our hypothesis, we performed a field experiment with four white cabbage cultivars. The cultivars (with seed supplier and if known the breeder) used in this study were Badger Shipper (Centre for Genetic Resources NL, University of Wisconsin US), Christmas Drumhead (Centre for Genetic Resources, NL), Lennox, and Rivera (both breeder and source Bejo seeds, NL). The four cultivars were selected based on their divergent glucosinolate profiles (chapter 2). Badger Shipper had relatively low concentrations of aromatic glucosinolates (gluconasturtiin) whereas Rivera had relatively high concentrations; the two other cultivars had intermediate concentrations. The setup of the experiment is described in detail in chapter 2. In short the field, comely used for cultivation of crops, was located near Wageningen, 51.95° north 5.64° east, The Netherlands, with the following soil characteristics N = 0.14%, C = 1.7%, C/N= 12, particle size distribution: <2 µm 5%, 2–63 µm 79%, >63 µm 19% . The field consisted of 32 plots (eight plots per cultivar), separated by 6 m wide grassland strips. On each plot one of the four cultivars was randomly assigned. The plots consisted of 49 plants in 7x 7 grids separated by at 75 cm distance. Plants were planted as three-week old plantlets pre-grown in a greenhouse on Lentse-potgrond (Horticoop, the Netherlands). The trial started on April the 28th, 2008. The field was weeded on a monthly basis and fertilized twice. On September 8th the roots of two plants per plot were harvested. These roots were pooled and half of the root material was used for glucosinolate analyses by HPLC as previously described by van Dam and Oomen (2008). The other pooled half was used for extraction of nematodes as described below.

Soil organism sampling

Soil organisms were sampled at two time points. The initial sampling took place on April 27, one day prior to planting, and represents the initial abundance of soil organisms in the field plots. The final sampling took place on September 8, 133 days after planting of the cultivars, at the same time as root samples for glucosinolate analysis were collected.

Microorganism community analysis

Initial samples of microorganisms, collected before planting of the cultivars, were obtained by pooling two soil cores per plot of 1.5 cm ø, which were collected from the top 10 cm of soil. Final samples, 133 days after planting of the cultivars, were obtained by sampling the soil directly adherent to the roots (rhizosphere soil) from the same two plants from which we collected root material for glucosinolate and nematode analysis. We analyzed the soil of six plots per cultivar. All samples were immediately put on ice, and transported to the laboratory before storage at -80˚C. To analyze the microbial diversity we used PCR-DGGE. DNA was isolated using a PowerSoil DNA isolation kit (MO BIO Laboratories, CA, USA) following the producer's instructions, with the addition of an initial step of 10 minutes of bead beating. DNA was eluted in 100 µl MilliQ. PCR was performed with universal 968-GC and 1378 primers for bacteria (Muyzer, Dewaal *et al.*, 1993) and FR1-GC and FF390 primers for fungi (Vainio & Hantula, 2000). For both PCR reactions 1 µl of total DNA was added to 24 µl PCR reaction (17.6 µl MilliQ water, 2.5 µl 10 x PCR buffer (Roche Diagnostics, Germany) 2.5 µl of 2 mM dNTP (Amersham Biosciences, US), 0.4 µl taq-Polymerase (Roche Diagnostics, Germany) and 0.5 µl of both primers. For DNA amplification we followed the protocol of Yergeau *et al.* (2007) for bacteria and Vainio and Hantula (2000) for fungi. Both amplifications were performed with a thermal cycler PTC-200 (Bio-Rad, US) and successful amplification was confirmed by electrophoresis of the PCR product (2 µl). Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described previously (Yergeau *et al.*, 2007) in a Universal Mutation Detection System (Bio-Rad, US). Gels were stained in ethidium bromide and photographed with an Imago apparatus (Gentaur, Belgium).

Nematode extraction and analysis

Nematodes were extracted from the roots only at the final sampling. For this we used half of the pooled root material (see glucosinolate analysis above). Root material was stored in a cooling box during transport to the laboratory before storing the samples for 18 hours in a climate chamber at 4˚C. The nematodes were extracted with the help of a mistifier according to van Bezooijen (2006). Numbers of root-inhabiting nematodes are expressed per g dry root. For nematodes from the soil we collected eight sub-samples per plot by removing soil cores (see above). All sub-samples were pooled to produce

one replicate sample per plot. For the final sampling soil nematodes were collected, using similar soil cores, next to the main root. Nematodes were extracted from soil by Oostenbrink elutriator and conserved with 4% formaldehyde before identification and expressed as numbers per 100 g dry soil. For both the nematodes from the root and soil we counted all nematodes present in the samples. Subsets of 150 nematodes were identified according to Bongers (1988) with the help of an inverted microscope to estimate the distribution over the families/genera. Identified nematodes were grouped on the basis of their feeding guilds (Yeates, Bongers *et al.*, 1993). Additionally weight of the nematodes was calculated by the formula $W = (w^2 \cdot l) \cdot (1.6 \cdot 10^6)^{-1}$ (Freckman, 1982), whereby W is the wet weight, w is the width of the nematodes at the widest point, and l is the length of the nematodes in µm. Lengths were taken from Bongers (1988).

Mites and Collembola extraction and analysis

Mites and Collembola were sampled and extracted simultaneously. Per plot we sampled one soil core of 10 cm depth and of 8.5 cm ø. This was done in the middle of the plot at the initial sampling and next to the root system of one of the cabbage plants at the final sampling. Extraction was performed following the protocol of Holtkamp *et al.* (2008). The Collembola were identified to the family level according to Fjellberg (1980) whereas for mites only total abundances were recorded. As microarthropods were not divided into functional groups we could not calculate the biomass (Holtkamp *et al.*, 2008). Therefore we expressed the abundances as numbers and analyzed these. Numbers of mites and Collembola were counted using a stereomicroscope.

Earthworms and enchytraeids

For enchytraeids one sample per plot was taken, both at the initial and at the final sampling. Soil cores (10 cm depth and 5 cm ø) at final sampling were taken near the roots. Extraction was done using a Baermann funnel (O'Connor, 1957). For earthworms one soil monolith of 20 x 20 cm and 10 cm depth was excavated per plot, both at the initial and final sampling. The earthworms were collected by hand-sorting.

Enchytraeids were counted using a stereomicroscope and their length, width, and surface area was measured with the program Axio vision (Carl Zeiss imaging solutions) and their weight was calculated by the formula log W = -0.6056 + 1.8546 log L (Abrahamsen, 1973b), where L is the length and W the wet weight. Earthworms were indentified to the species level according to Christian and Zicsi (1999), their length was measured, and their wet weight calculated by the formula log W = -2.255 + 2.071 log L (Abrahamsen, 1973a). Weights are expressed on the basis of 100g dry weight of the soil.

Statistical analysis

Glucosinolate data

Glucosinolate profiles of the four cabbage cultivars, in each of the plots, previously described in chapter 2, were used in the current study to test whether variation in these profiles could explain variation in the abundance of microorganisms, nematodes in the roots, nematodes in the soil, microarthropods, and earthworms. Analyses were based on plot means for glucosinolate concentrations and the abundances of the different species per plot for soil organisms.

To analyze whether glucosinolates can explain differences in diversity we used a multivariate method, redundancy analysis (RDA). This model allows multiple variables, in our case the glucosinolates, to be tested for their explanatory power. In cases where glucosinolates could significantly explain the abundance data we used a forward selection procedure to identify the glucosinolates with the highest explanatory power within the profile. For the microorganisms we used canonical correspondence analysis (CCA) instead of a RDA because of the binarity of the data. Monte Carlo permutations (MCP) were used to test the significance of differences along the first axis and all other axes. All RDA/CCA analyses were performed in CANOCO 4.5 for Windows (Biometris, Wageningen, NL).

DGGE data

DGGE gel banding patterns were analyzed using the Image Master 1D program (Amersham Biosciences, the Netherlands). The resulting binary matrices were used as 'species' presence–absence matrices. To test for the effects of cultivar multivariate tests of significance were carried out using CCA in CANOCO. The significance of each treatment was tested by 999 permutations on both the first and second axis.

Nematode data

Analysis of Covariance (ANCOVA) was used to analyze plot and cultivar effects on nematode biomass. The biomass of the nematodes at the first sampling was used as a continuous predictor, cultivar as a categorical variable, and the biomass of the nematodes at the second sampling as the dependent variable. This analysis was performed to correct for possible spatial heterogeneity at the start of the experiment. Data was log transformed, if needed, to achieve normality or homogeneity of variances.

To assess whether our sampling scheme was sufficient at both the initial and final sampling to allow further analyses of the nematode community we estimated sampling success (*i.e.*, the number of sampled families out of the estimated real number of families). We applied a non-parametric first order jackknife approach using the program EstimateSWin820 (University of Connecticut, US).This analysis revealed that our sampling success was comparable between initial sampling, before the cabbages were planted, and final sampling (Supplementary **Table S3.1).** Therefore we analyzed both samplings simultaneously. To analyze the differences in nematode abundances among cultivars we used a RDA model with MCP as test of significance. To correct for spatial heterogeneity, plot identity was additionally coded as a dummy variable and defined as co-factor in the analysis, an approach similar to linear polynomial contrasts (Lepš *et al.*, 2003).

Mites, Collembola, enchytraeids, and earthworms

ANCOVA was used to analyze abundances for mites and Collembola, and enchytraeids, as well as earthworm biomass as described above. t-Tests or Wilcoxon rank-sum tests were used to compare initial and final samplings. RDA as described above was used for differences in diversity of Collembola or earthworms between cultivar, as these groups were identified at the family and species level, respectively.

Results

Nematodes in the roots

The majority of nematodes in the roots belonged to the genus *Pratylenchus*. The number of root nematodes differed vastly between cultivars. Badger Shipper had higher numbers of nematodes than the other cultivars (**Table 3.1**). Glucosinolate profiles of the cultivars significantly correlated with nematode abundance (RDA MCP P <0.01). Following forward selection to identify the most explanatory glucosinolates, the model only retained gluconasturtiin, which was negatively correlated (P =0.004, F =6.8), and glucoerucin, which was positively correlated (P =0.002, F =13.8) with *Pratylenchus* abundances (Supplementary **Figure S3.1**).

Table 3.1: Number of nematodes recovered from the roots of the cultivars. Different letters denote significant differences by one-way ANOVA and Tukey's test at P <0.05

	Total nr.	*Pratylenchus*
Badger Shipper	156 ± 65 a	151 ± 64 a
Christmas Drumhead	10.0 ± 3.6 b	7.3 ± 3.4 b
Lennox	9.9 ± 4.1 b	8.9 ± 4.2 b
Rivera	2.9 ± 0.6 b	1.7 ± 0.5 b

Nematodes in the soil

RDA did not reveal cultivar-specific effects on nematode communities in the soil (MCP P >0.1,). In addition, glucosinolates were not associated with nematode abundances (RDA MCP P >0.5). The *Pratylenchus* recorded in the soil tended to be negatively correlated with root concentrations of gluconasturtiin, as observed for nematodes recorded in the roots, but glucosinolate effects on the soil nematode community were not significant (**Figure 3.1**, glucosinolates by nematode community RDA MCP P =0.066).

The cultivar Badger Shipper supported a higher biomass of plant-feeding nematodes in the soil (significant effect of Badger Shipper vs. Lennox and Rivera, (**Table 3.2**). The biomass of other feeding guilds did not differ between cultivars (**Table 3.2**).

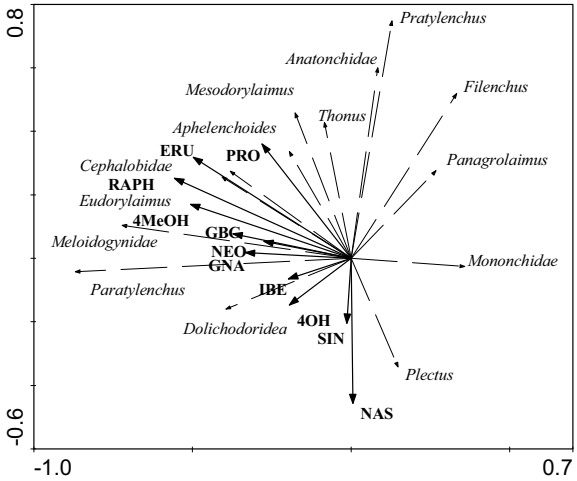

Figure 3.1: Associations between glucosinolates and the nematode community in the soil, Axis one explains 31.2% of the variation axis 2 explains 15.6%. Abbreviations: IBE; glucoiberin, NAS; gluconasturtiin, SIN; sinigrin, 4OH; 4-hydroxyglucobrassicin, GNA; gluconapin, NEO; neo-glucobrassicin, GBC; glucobrassicin, NEO; neo-glucobrassicin, 4MeOH; 4-methoxyglucobrassicin, RAPH; glucoraphanin, ERU; glucoerucin, PRO; progoitrin

Table 3,2: Average wet weight or average number of individuals ± standard error, expressed per 100 g dry weight soil^{-1}. Different letters denote significant differences by ANCOVA analysis and Tukey's test at P <0.05

	Badger Shipper	Christmas Drumhead	Lennox	Rivera
Nematodes in the soil				
Total biomass in µg wet	1815 ± 302 a	1686 ± 466 a	1018 ± 144 a	1088 ± 131 a
Plant-feeding biomass in µg wet	255 ± 41 a	186 ± 33 ab	161 ± 13 b	167 ± 32 b
Hyphal-feeding biomass in µg wet	2.55 ± 0.64 a	1.27 ± 0.51 a	2.31 ± 0.76 a	2.06 ± 0.91 a
Bacterial-feeding biomass in µg wet	1298 ± 223 a	1370 ± 473 a	772 ± 111 a	731 ± 111 a
Animal-predator biomass in µg wet	227 ± 68 a	99 ± 43 a	70 ± 49 a	170 ± 59 a
Omnivores biomass in µg wet	30.7 ± 5.1 a	28.9 ± 10.7 a	13.6 ± 6.7 a	19.2 ± 5.80 a
Mites				
Number of total individuals	1.93 ± 0.18 a	1.82 ± 0.27 a	1.48 ± 0.35 a	1.18 ± 0.25 a
Springtails				
Number of total individuals	2.09 ± 0.36 a	2.20 ± 0.45 a	2.62 ± 0.23 a	3.01 ± 0.71 a
Number of Onychiuridae	0.53 ± 0.15 a	0.46 ± 0.15 a	0.35 ± 0.12 a	1.00 ± 0.44 a
Number of Poduridae	0.08 ± 0.08 a	0.12 ± 0.06 a	0 ±0 a	0.05 ± 0.05 a
Number of Entomobryidae	0.71 ± 0.21 a	1.09 ± 0.33 a	0.78 ± 0.14 a	0. 96 ± 0.68 a
Number of Isotomidae	0.77 ± 0.25 a	0.53 ± 0.20 a	1.49 ± 0.63 a	1.00 ± 0.21 a
Earthworms and Enchytraeids				
Earthworm biomass in µg wet	15004 ± 3509 a	14886 ± 4327 a	6505 ± 1116 a	4756 ± 962 a
Enchytraeids biomass in µg wet	724 ± 329 a	1843 ± 669 a	696 ± 338 a	735 ± 242 a

Microorganisms

At the initial sampling, bacterial communities did not show a separation according to plots assigned to different cultivars (1st CCA axis MCP P >0.9 2nd axis MCP P >0.9). Thus, there were no plot differences prior to the experiment. At the final sampling, the bacterial composition did not show any response to cultivars (1st axis MCP P >0.5, 2nd axis MCP P >0.2). The community composition of fungi also did not differ among cultivars (initial sampling: 1st axis MCP P >0.3, 2nd axis MCP P >0.1; final sampling: 1st axis MCP P >0.5, 2nd axis MCP P >0.2). There was no correlation between glucosinolate profiles and DGGE patterns of bacteria (CCA MCP P >0.1) or of the fungi (CCA MCP P >0.5).

Soil microarthropods

Numbers of mites significantly increased between initial and final sampling (t-test P <0.05, 0.79 ± 0.08 vs. 1.61 ± 0.14 individuals per 100g dry soil respectively). In contrast, numbers of Collembola did not differ between the first and second sampling (t-test P >0.5, 1.9 ± 0.2 vs. 2.4 ± 0.2 individuals per 100g dry soil respectively).

The total numbers of Collembola and mites were not different between the four cultivars (**Table 3.2**). Neither the mites nor the Collembola showed any correlation with glucosinolate profiles (both mites and Collembola RDA MCP P >0.1) or cultivars (both RDA MCP P >0.5).

Enchytraeids and earthworms

The estimated biomass of enchytraeids and earthworms significantly increased between initial and final sampling dates (enchytraeids: t-test P < 0.05, 92 ± 36 µg vs. 1000 ± 221 µg per 100g dry soil respectively; Earthworms: Wilcoxon rank-sum test P <0.05, 4845 ± 642 µg vs. 10288 ± 1549 µg per 100g dry soil respectively). However, neither the magnitude of this increase, for both enchytraeids and earthworms, nor the diversity of earthworms was affected by cultivar (biomass **Table 3.2**, diversity RDA MCP P >0.1). Additionally, earthworms diversity was not correlated with glucosinolate profiles (RDA MCP P >0.05).

Discussion

Nematodes

Our results show that there is a clear cultivar effect on migratory endoparasitic nematodes of the genus *Pratylenchus* in the roots. The cultivar Badger Shipper supported more individuals of this genus than the other three cultivars. This positive effect is likely due to the lack of gluconasturtiin in roots of the Badger Shipper cultivar. The toxicity of gluconasturtiin on soil organisms, including plant parasitic nematodes, in Brassicaceae has been described earlier in greenhouse environments (Potter *et al.*, 1998; Potter *et al.*, 2000). *Pratylenchus* abundances in the present study were positively correlated with glucoerucin. This contradicts in vitro tests with root-knot nematodes in which their abundances were reduced when exposed to breakdown products of glucoerucin (Lazzeri *et al.*, 2004). One possible explanation for these contrasting results is that *Pratylenchus*, in contrast to root-knot nematodes, might be resistant to this specific glucosinolate. Aboveground positive correlations between specific glucosinolates and particular insect species have been observed before (Moyes, Hamish *et al.*, 2000). To our knowledge, our result is the first report of such a positive correlation for a belowground herbivore. An additional explanation might be that there is a trade-off between the production of gluconasturtiin and other glucosinolates. Badger Shipper may have reallocated its resources from the production of gluconasturtiin to glucoerucin. This is supported by the observation that glucoerucin is occurring in twice as high concentrations in this cultivar when compared to the three other cultivars (chapter 2). The biomass of plant-feeding nematodes outside the roots was also higher under the Badger Shipper cultivar, which is a likely consequence of the high reproduction rates of *Pratylenchus* inside the roots of Badger Sipper, and subsequent migration into the rhizosphere.

Higher trophic levels and microorganisms

In principle, the higher numbers of nematodes associated with the Badger Shipper cultivar both outside and inside the roots could have resulted in higher abundances of other groups of organisms that feed on these nematodes in the soil of this cultivar as well. However we found no significant effects on higher trophic levels, as the numbers or the biomass of mites and carnivorous nematodes were not affected by cultivar. The lack of an effect on these higher trophic level organisms may be due to the relatively low biomass of root-feeding nematodes in and around the roots in relation to other soil organisms. In any case, cultivar effects on the genus *Pratylenchus* did not cascade up to higher trophic levels.

That belowground higher trophic levels were not affected by cultivars is in contrast to what has been previously recorded on the same cultivars for aboveground higher trophic levels (Poelman *et al.*, 2009). In that study, the abundances of natural enemies aboveground were affected by differences in glucosinolate profiles. The difference in the effects that glucosinolates and their breakdown products have on aboveground and belowground communities might be explained by considering differences between these environments. First, belowground predators are less mobile than aboveground predators and parasitoids, so that the area at which belowground natural enemies can be affected may be much smaller. Second, volatile breakdown products of glucosinolates are less mobile in the soil (Gimsing *et al.*, 2009). Defense against natural enemies by volatile breakdown products of glucosinolates may therefore be less effective belowground than aboveground. Therefore plants may, at least in our model system in which the roots exhibit a strong direct defense, mainly rely on constitutively direct instead of inducible indirect defenses belowground (van Dam *et al.*, 2009).

The reduced exposure to glucosinolates or their breakdown products could also explain why detritus feeders were not affected. Decomposers, such as earthworms or enchytraeids, could potentially be influenced by exposure to glucosinolates either by the ingestion of senescent plant material or by exposure to exudation from roots. However, the biomass of organisms in the decomposer food web was not affected by cultivar, nor was their species composition correlated with glucosinolates. Even though microorganisms feed

on a variety of food sources (*i.e.* living and dead plant material and root exudates) we still found no differences in the microorganism community between the cultivars or a response of the microorganisms to the glucosinolate profiles. This contrasts with previous studies recording that *B. napus* and *A. thaliana* chemotypes with different glucosinolate profiles influenced the microorganism community (Rumberger *et al.*, 2003; Bressan *et al.*, 2009). Using identical bacterial primers as used in our study, Rumberger *et al.* (2003) found that 2-phenylethyl-isothiocyanate, the breakdown product of gluconasturtiin, influenced the bacterial community in roots and rhizosphere of *B. napus*. However, these effects only occurred at extremely high concentrations of 2-phenylethyl-isothiocyanate, above 1300 pmol/g soil. These high concentrations are unlikely to occur in the roots or rhizosphere of white cabbage. Moreover, in *B. napus* gluconasturtiin is the most abundant glucosinolate (Sarwar *et al.*, 1998b) whereas gluconasturtiin is only the fourth most dominant glucosinolate in the cultivars used in this study (chapter 2).

In conclusion, we found that cultivar variation only affected plant-feeding nematodes. These soil organisms interact directly with living plant roots. No differences were recorded for soil microbes that potentially feed on dead plant materials and other trophic levels in the soil food web. This indicates that variation in chemical defense profiles causing differences in herbivore abundances do not necessarily trickle up to higher trophic levels in the soil food web.

Supplementary material

Table S3.1: Results of the jackknife analysis to estimate the expected species diversity form the recorded species diversity.

	Species recorded	Jackknife lower estimate	Jackknife higher estimate	Percentage of species found
Initial sampling				
Badger Shipper	27	31.71	36.29	79.41
Christmas Drumhead	26	29.1	31.66	85.58
Lennox	24	25.35	27.91	90.12
Rivera	28	34.19	39.31	76.19
Final sampling				
Badger Shipper	23	24.63	28.37	86.79
Christmas Drumhead	24	25.63	29.37	87.27
Lennox	23	24.35	26.91	89.74
Rivera	22	22.6	24.9	92.63

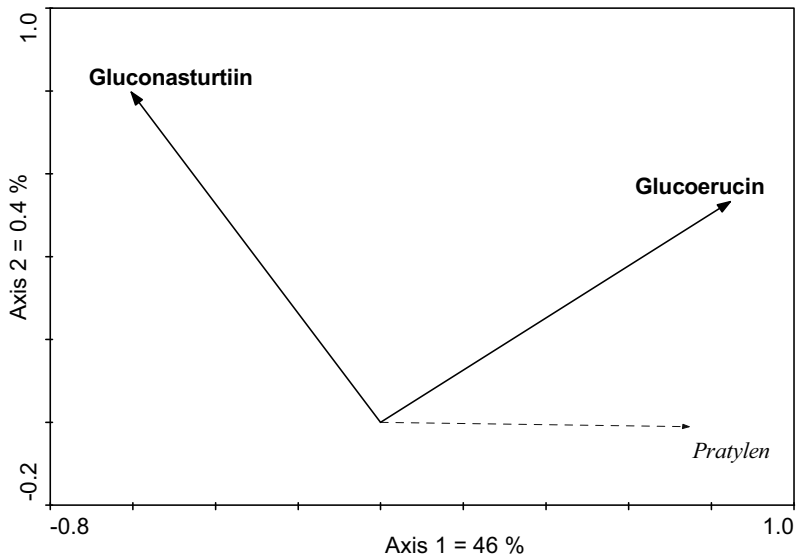

Figure S3.1: Glucosinolates which are found to significantly explain the effect of on the genus *Pratylenchus* by RDA forward selection. Other nematode species were removed as these had a relatively small contribution to the model (less than 0.2 loading)

Chapter 4

Glucosinolate concentrations and profiles in roots and root exudates: why plant glucosinolate profiles have low predictive power for effects on soil organisms

Patrick Kabouw, Nicole de Goeij, Wim H. van der Putten, Arjen Biere, and Nicole M. van Dam

Abstract

Aims: Root exudates, although important as primary resources, can also negatively influence soil biota if they contain toxic or detrimental secondary plant metabolites. Glucosinolates, a group of secondary metabolites commonly found in Brassicaceae can negatively affect soil biota at different trophic levels. These effects have been assessed in laboratory studies and in biofumigation experiments in which entire glucosinolate containing Brassica plants are incorporated into the soil. However, the effects of glucosinolates and their breakdown products as found in laboratory settings do not correspond to the effects that intact plants and their root exudates have on rhizosphere communities. We hypothesized that this may be due to a lack of correlation between glucosinolate concentrations and profiles in the root exudates and those in roots.

Methods: To test this hypothesis we used rhizotron systems to collect root exudates of different brassicaceous species and cultivars. Correlations between glucosinolates in the exudates and those in the roots were analysed.

Results: Total glucosinolate concentrations of roots and root exudates were correlated, but that this was not the case for glucosinolate profiles. The inter-specific variation recorded in root glucosinolate profiles was not reflected by similar inter-specific differences in the exudates. This lack of correlation in profiles might, at least in part, be due to different exudation or degradation rates between the glucosinolates. Indeed, we found that the degradation rate of glucosinolates is partly dependent on their biosynthetic origin.

Conclusions: The lack of correlation between the glucosinolate profiles in the root exudates with those in the roots could explain why organisms that are directly associated with the plant roots are more likely to be affected by intra- and inter-specific variation in glucosinolate profiles than organisms of higher trophic levels.

Keywords; Intraspecific variation, Interspecific variation, Soil organisms, Rhizosphere, Rhizotrons

Introduction

Root exudates are important in shaping interactions between plants and soil biota (Lammers, Arbuckle-Keil *et al.*, 2009; Dennis, Miller *et al.*, 2010; Hannula, de Boer *et al.*, 2010). The outcome of these interactions may range from positive to negative, depending on the composition of the exudates and on the functionality of the responding soil communities. The exudation of plant primary metabolites, such as sugars or amino acids, generally promotes the development of soil communities by providing nutrients to microbes in the rhizosphere (Jones *et al.*, 2009; Lesuffleur *et al.*, 2010). On the other hand, toxic secondary metabolites in the exudates can negatively influence members of the soil community (Scheidemann *et al.*, 1997; Rumberger *et al.*, 2003; Bais *et al.*, 2006; Wurst *et al.*, 2010b). There is an implicit assumption that root exudate composition is similar to that of roots. In the present study, we test this assumption by determining how root exudate composition correlates to that of plant roots and how this affects levels of plant secondary metabolites in the rhizosphere.

Here we investigate glucosinolates, which are a class of well-studied secondary metabolites that can negatively influence soil communities. Glucosinolates occur primarily in the family Brassicaceae. They are water soluble organic compounds that hydrolyze to (iso)-thiocyanates, nitriles, or oxazolidine thiones when they come into contact with the enzyme myrosinase (Mithen, 2001). Glucosinolates and their hydrolysis products are generally known to be toxic or detrimental to a variety of soil organisms such as Collembola, nematodes, fungi, and bacteria when tested in laboratory experiments (Angus *et al.*, 1994; Brown *et al.*, 1997; Potter *et al.*, 1998; Rumberger *et al.*, 2003; Jensen *et al.*, 2010). Due to this toxicity, members of the Brassicaceae are frequently used as biofumigation crops that are ploughed under to suppress soil-borne pathogens such as phytopathogenic fungi and plant-parasitic nematodes (Kirkegaard *et al.*, 1998; Sarwar & Kirkegaard, 1998a; Gimsing *et al.*, 2009).

Glucosinolate concentrations and profiles (*i.e.* the relative contribution of the different types of glucosinolates to the total concentration) in plants are known to differ both within and between plant species. Intra- and inter-specific glucosinolate variation in the aerial parts can affect herbivores, as well as higher trophic level organisms (Harvey, van Dam *et al.*, 2003; Gols *et al.*, 2007; Müller, 2009). Belowground intra- and inter-specific variation in

glucosinolate concentrations and profiles are also commonly recorded (Kirkegaard *et al.*, 2000). Similar to what has been found for the aerial parts, belowground intra- and inter-specific variation in glucosinolates can affect the abundance of microorganisms and plant-parasitic nematodes, which are directly associated to the plant (Bressan *et al.*, 2009). However, higher trophic level organisms in the rhizosphere food web, as well as organisms not directly associated to the plants roots such as detrivorous organisms, do not appear to be affected by inter- and intra-specific variation in root glucosinolates (Rumberger *et al.*, 2003; Bressan *et al.*, 2009).

We tested two hypotheses to explain why belowground higher trophic level organisms and those not directly associated with roots do not respond to intra- and inter-specific variation in root glucosinolates. The first hypothesis is that there is no correlation between glucosinolate concentrations and/or profiles in the roots and those in root exudates. Even though this has been proposed as a possible explanation before (Bressan *et al.*, 2009), it has never been experimentally tested. The second hypothesis is that aerial parts of the plants show more variation in glucosinolate concentrations and/or profiles than belowground plant parts. Larger intra- and inter-specific variation in glucosinolate profiles can result in stronger effects on organisms. If intra- and inter-specific variation is indeed larger in aerial parts than in belowground parts this could potentially explain why differences in glucosinolates affect higher trophic levels aboveground but not belowground.

To address these hypotheses we performed four experiments. In two experiments we determined whether glucosinolate concentrations and profiles in root exudates of different Brassicaceae species and cultivars are correlated with those in roots. We performed these tests in rhizotrons in combination with micro-suction cups. Prior studies have shown that micro-suction cups are suitable for the extraction of heavy metals, sugars, and amino acids present in exudates (Gottlein, Hell *et al.*, 1996 ; Puschenreiter, Wenzel *et al.*, 2005; Shen & Hoffland, 2007; Neumann, George *et al.*, 2009). In addition, we correlated glucosinolate concentrations and profiles in shoots with those in the roots. This allowed us to test if intra- and inter-specific variation in root glucosinolates was indeed lower than in shoots. In a third experiment we investigated the persistence of glucosinolates in the soil in the presence and absence of micro-organisms to gain insight in the fate of exuded glucosinolates (glucosinolate degradation experiment). Finally, we assessed how well the concentrations and profiles observed in the rhizotrons reflect those in less physically constrained root growth conditions by comparing root

glucosinolates of plants grown in rhizotrons with those of plants grown in pots (pot experiment).

Material and methods

Rhizotron experiments

The rhizotrons used in this study have been described in detail in Gottlein *et al.* (1996). Briefly, they consist of plastic plates of which the top plate (32.5 by 20.5 cm) contains small holes (0.8 mm ø) spaced at 5 mm distances. These holes allow the insertion of micro-suction cups close to the roots. The lower plate (same dimensions as above) is transparent, which allows the monitoring of root locations. When not collecting root exudates, the transparent plate is covered by a black plastic plate (same dimensions as above) to block light entry. The dimensions inside the rhizotrons are 31.5 cm (height) by 19.5 cm (width) by 1.5 cm (depth) with a total volume of 0.92 l. The rhizotrons are completely covered by aluminum foil to prevent light entry and to reduce evaporation. Rhizotrons are placed under an angle of approximately 70 degrees by suspending them between two clamps. As the recovery test (see below) revealed that sand resulted in the highest recovery of artificially added glucosinolates, we selected sand as the most suitable substrate for the rhizotron experiments.

Two rhizotron experiments were performed to test for associations between root and rhizosphere glucosinolates across different brassicaceous species and cultivars. In the first experiment we used one cultivar of *Brassica oleracea* and two cultivars of *Raphanus sativus* (**Table 4.1**) with six replicate plants each. In the second experiment we used three cultivars of *B. oleracea* and one cultivar of *Sinapis alba* with five replicate plants each (**Table 4.1**). We used both cultivars that are intended for human consumption and cultivars used as green manure. We expected these cultivars to have different glucosinolate concentrations and/or profiles (Brown *et al.*, 1997). Seeds of the cultivars were germinated in a growth cabinet, in plastic containers filled with a layer of glass beads (~1 cm) and demineralized water. The growth cabinet had a day/night period of 16:8 h and a temperature of 25:15 °C, respectively. After two weeks seedlings were transferred to the rhizotrons that were kept in a greenhouse at: 25:15 °C (day/night), 16:8 h, and a minimum photoactive radiation (PAR) of 225 μmol m^2 s^{-1}. During both rhizotron experiments we

sampled root exudates twice. The first sampling occurred one week prior to harvest of the plants, and the second sampling directly prior to harvest of roots and shoots, thus allowing direct correlations between the glucosinolates in the plants and in the root-exudates. Two rhizotrons without any plants served as a negative control.

Table 4.1: Species and cultivars used in the two rhizotron and pot experiments

Cultivar	Species	Intented for	Breeder	First or second root-box experiment
Rivera	White Cabbage	Human comsuption	Bejo seeds	First and second
Terranova	Raphanus sativus	Green manure	Joordens seeds	First
Doublet	Raphanus sativus	Green manure	Joordens seeds	First
Architect	Sinapis alba	Green manure	Joordens seeds	Second
Jetma	White cabbage	Human comsuption	Rijk Zwaan	Second
Opaal	Cauliflower	Human comsuption	Rijk Zwaan	Second

The micro-suction cups (Frits Meijboom, Wageningen, The Netherlands) that were used to collect the exudates have been described previously (Shen *et al.*, 2007). Briefly, the tips of the micro-suction cups through which exudates are collected have a length of 6 mm, a circumference of 1.1 mm, and a pore size of <0.2 µm to prevent microbes from passing. For collection of exudates these micro-suction cups were inserted in holes at a distance of 7 to 10 cm from the bottom of the rhizotrons, where most roots were observed. The micro-suction cups were inserted adjacent to the root system so that the roots surrounded the tip of the micro-suction cups. To collect the exudates from the rhizosphere the pressure inside the micro-suction cups was reduced to around -90 kPa by applying negative pressure with a 10 ml syringe. The syringe was prefilled with 1ml 70% MeOH and was cooled to -20°C so that glucosinolates in the exudable fraction would not be hydrolyzed by myrosinase. One hour prior to inserting the micro-suction cups, the soil in the rhizotrons was calibrated to contain 14% moisture (dry mass/soil) using tap water.

Glucosinolate recovery

Prior to the rhizotron experiments we tested the recovery of glucosinolates from different substrates. Two substrates were used; river sand (particle size distribution: <125 µm 13%; 125 -250 µm 83%; >250 µm 4%) and a loamy, sandy mineral soil (Planken Wambuis; 52.06° N 5.75° E, The Netherlands, N = 0.13%, C = 2.1%; particle size distribution: <2 µm 3%; 2 - 63 µm 17%; >63 µm 80%). Initially we also included a clay soil but we were unable to recover any liquid from this soil with the micro-suction cups, probably due to the high water retention potential of this soil. To 40 grams (wet weight) of substrate we added 1 ml of a glucosinolate mix that was created by extracting intact glucosinolates from twelve different white cabbage cultivars of known glucosinolate composition (chapter 2). Total glucosinolate concentration of the mix was 358 ± 4.2 µM distributed over eight glucosinolates. Ten replicates were used per soil type. Glucosinolates were left for 30 min in the substrates before extraction. According to Gimsing *et al.* (2005) this time period allows for chemical reactions with the soils without substantial microbial degradation or hydrolysis. Soil liquid was collected using the micro-suction cups, transferred to microcentrifuge tubes and stored at -20°C before glucosinolate extraction (see below). The analyses revealed that the recovery of the added glucosinolates with the micro-suction cups was higher in river sand than in mineral soil although the difference was not significant due to the high variation in the glucosinolate measurements (sand; average recovery 102 ± 12% standard error, mineral soil 73 ± 18%, Wilcoxon test Z =1.24 P >0.10). Nevertheless, as sand had on average the highest recovery, we opted for sand as the most suitable substrate type.

Glucosinolate degradation in the presence or absence of microorganisms

In this experiment, we tested the degradation of glucosinolates over time and the role of micro-organisms in the degradation of glucosinolates. The setup of this experiment was similar to that of the recovery experiment described above. One ml of glucosinolate solution was added to 40 grams (wet weight) of mineral soil in 50 ml laboratory tubes. The soil was either sterilized, by gamma radiation (Isotron, Ede, The Netherlands), or non-sterilized. For the degradation experiment we opted for the mineral soil as we expected that this soil would have a more diverse microorganism community than river sand. The tubes with the soil were covered with aluminum foil and capped with

paper laboratory stoppers, to allow limited air flow while maintaining a constant moisture level. The tubes were held for 24, 72, and 120 hours under greenhouse conditions (as described above) before extraction. Glucosinolates were extracted directly from the soil (so without the micro-suction cups) according to Gimsing *et al.* (2005). Degradation over time is expressed as a percentage of added glucosinolates. At all time points and for both sterilization treatments, ten replicates were analyzed.

Pot experiment

We also checked whether root glucosinolate concentrations and profiles of plants grown in the rhizotron experiments are representative for those grown under more commonly practiced and spatially less constrained conditions. Here the same cultivars as used in the rhizotron experiments were grown in pots where the roots could distribute more evenly and also had a larger volume (1.1l). Seeds were germinated under identical conditions as described under "rhizotron experiments". Seedlings were transferred to the pots filled with similar river sand as used for the rhizotron experiments before relocation to a greenhouse with identical conditions as the rhizotron experiments. After six weeks the roots and shoots of these plants were harvested, frozen, and their glucosinolates analyzed.

Glucosinolate analyses

Glucosinolates in roots and shoots were extracted according to van Dam and Oomen (2008). For glucosinolate extraction from the soil we followed the protocol recommended by Gimsing *et al.* (2005) with some minor alterations. In contrast to the above protocol we used 2 grams (dry weight) of soil and 2 x 1 ml 70% methanol to ensure optimal extraction. For the extraction of glucosinolates from the exudable fraction we used a modified protocol. First, the syringe used for extraction was weighed before and after collection to determine the amount of exudation extracted from the rhizotrons. The entire content, which included the pre-filled methanol from the syringe, was transferred into a microcentrifuge tube and heated to 90°C to deactivate myrosinase activity. This solution was transferred to a Sephadex A-25 column to bind the glucosinolates. Purification of the glucosinolates and HPLC analysis of de-sulphated glucosinolates was also performed after van Dam *et al.* (2008).

Dilution factors on the HPLC were adjusted to correct for the amount of liquid that was extracted from the rhizotrons.

Statistical analyses

To test for differences in glucosinolate degradation between sterilized and non-sterilized soils over time and between glucosinolates of different biosynthetic origin, a repeated measures ANOVA was used. If needed the data was log transformed to achieve normality and homogeneity of variances. All statistical analyses were performed in Statistica (Statistica, StatSoft, Tulsa, OK). Correlations were used to test for associations between glucosinolate concentrations in roots and shoots (here both the rhizotrons and the pot experiments were compared simultaneously) and between glucosinolate concentrations in roots and root exudates of the different species and cultivars.

To compare glucosinolate profiles between roots and shoots, and between plants grown in rhizotrons and pots, we used redundancy analysis models and, if appropriate, Monte Carlo Permutations (MCP). To examine whether inter- and intra-specific variation in glucosinolate profiles was consistent for plants grown in rhizotrons and pots we analyzed multivariate interactions by applying MCP in CANOCO (Biometris, Wageningen, NL). Ordination diagrams were made in CANOCO and were used to visualize the separation between the cultivars (*i.e.* the intra- and inter-specific variation) and between roots and shoots. The intra- and inter-specific differences of rhizotron and pot experiments were visualized in a single ordination diagram. For this, the data was centered and standardized (Lepš *et al.*, 2003).

Levene's tests for equality of variances were used to test whether the extent of glucosinolate variation in the roots differed from that in the leaves. We used Levene's tests as these are suitable for the analysis of non-normalized data. For a comparison of the extent of variation in total concentrations we used the raw data. To examine the extent of variation in profiles in roots and shoots we used the scores of the samples on the first two axes of the principal component analysis diagram and comparing these by Levene's tests.

Results

Comparison of glucosinolate profiles in roots and root exudates

A total of 14 glucosinolates were recorded in the roots of the six cultivars. However, only eight of these glucosinolates were recorded in the root exudates. Despite the discrepancy between the glucosinolate profiles of roots and root exudates, the total glucosinolate concentration of the root exudates at the second sampling was significantly correlated with the total glucosinolate concentration in the roots (**Figure 4.1**, P <0.01 R^2 =0.85). Interestingly, when glucosinolates were grouped according to their biosynthetic origin, percentages of glucosinolates in the roots were almost equally distributed over the three biosynthetic groups (**Figure 4.2**, ANOVA F =0.9 P >0.05; based on percentages according to biosynthetic origin, across all cultivars). In the root exudates, however, the distribution was not uniform; more aliphatic glucosinolates than indolyl and aromatic glucosinolates were found in the exudates (**Figure 4.2**; percentages based on biosynthetic origin: ANOVA F =7.5 P <0.001).

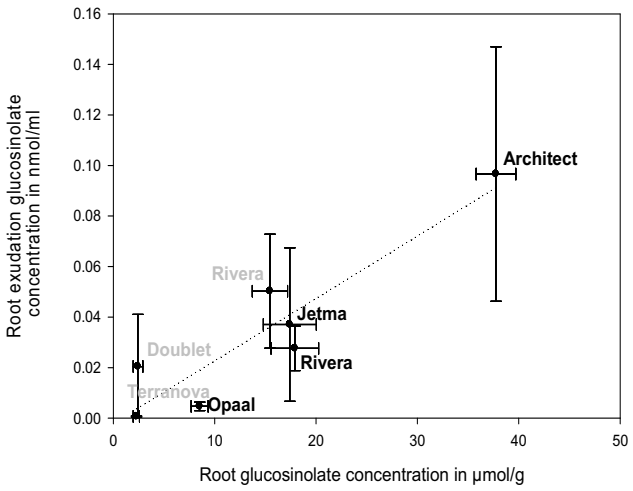

Figure 4.1: Correlations between total glucosinolate concentrations in roots and in root exudates (second sampling) for both rhizotron experiments. The cultivar Rivera is included twice as it was studied in both rhizotron experiments. Names of the cultivars in grey are from the first rhizotron experiment, in black from the second rhizotron experiment

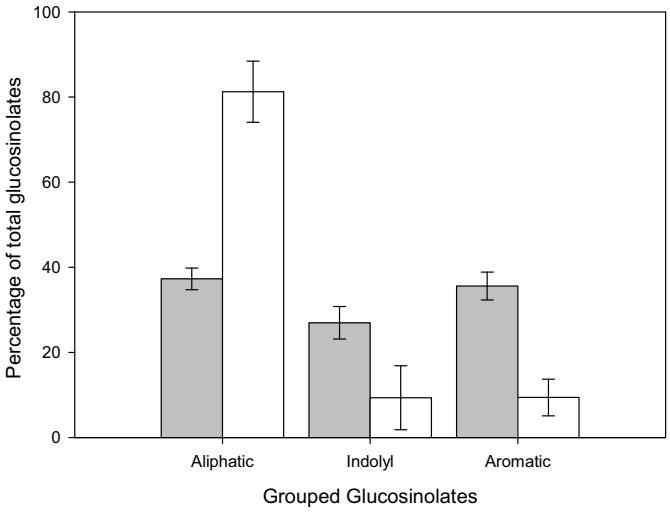

Figure 4.2 average (± standard error) distribution of the glucosinolates based on their biosynthetic origin. Grey bars: proportions in the roots; white bars proportions in root exudates

Root profiles differed significantly between the brassicaceous species. This inter-specific variation was larger than the intra-specific variation (see **Figure 4.3A & B**). In the first rhizotron experiment, the root glucosinolate profile of the *B. oleracea* cultivar Rivera was clearly separated from the *R. sativus* cultivars Terranova and Doublet (see **Figure 4.3A**, b, MCP F =9.7 P <0.05), mainly due to high concentrations of gluconasturtiin in Rivera. However, the cultivars of *B. oleracea* and *R. sativus* could not be separated based on their root exudate glucosinolate profiles (see **Figure 4.3A**, c for the first sampling and d for the second sampling, first sampling MCP F =1.8 P >0.1, second sampling MCP F =1.6 P >0.1). Consequently, there was a significant interaction effect between cultivar and sample origin (root vs. exudates; MCP F =74.7 P <0.05). The observed lack of inter-specific variation in root exudate glucosinolate profiles is likely due to the absence of gluconasturtiin in the exudates, which contributed to the inter-specific differences in the roots. In the second rhizotron experiment a similar trend was observed. Here the root glucosinolate profile of the *S. alba* cultivar Architect with its high glucotropaeolin concentrations differed from that of the three *B. oleracea* cultivars (see **Figure 4.3B**, b, MCP F =24.5 P <0.01). However this was not accompanied by a separation of Architect from the three *B. oleracea* cultivars based on the root exudate glucosinolate profiles (see **Figure 4.3B**, c for the

first sampling and d for the second sampling, first sampling MCP F =1.5 P >0.5, second sampling MCP F =4.6 P >0.05). Thus again an interaction effect was recorded (MCP F =11.5 P <0.05), probably due to the absence of glucotropaeolin from the exudable fraction.

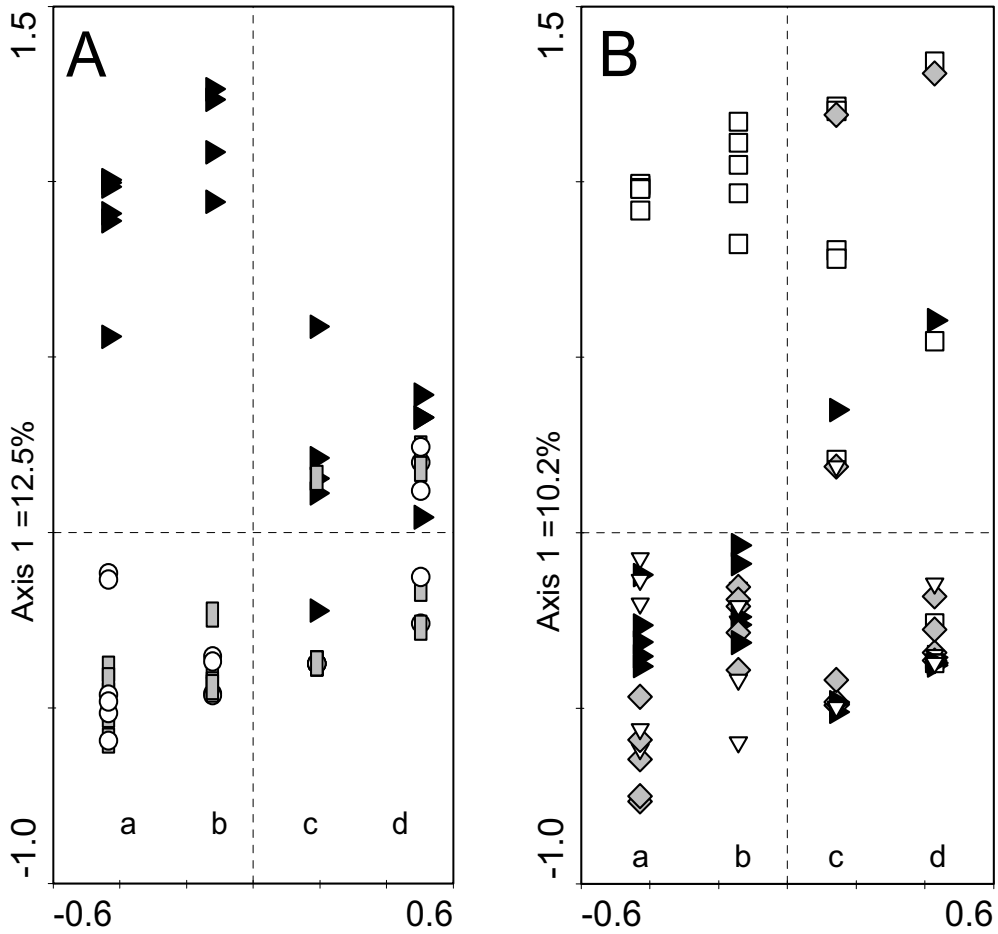

Figure 4.3: Redundancy analysis plots of inter- and intra-specific variation in glucosinolate profiles, with the first axis fixed for the different environments. A: samples from the first rhizotron experiment; B: samples from the second rhizotron experiment. The letters a-d within the graphs represent the four different combinations of sample origin and growth environment; a: root profile in the pot experiment; b: root profile in the rhizotron experiment; c: test for associations first root exudation profile; d: second root exudation profile ▸Rivera, ○ Terranova, ▌Doublet, □ Architect, ◆ Jetma, ▽ Opaal.

Comparison of glucosinolate profiles in roots and shoots

Total glucosinolate concentrations were significantly higher in roots than in shoots (Student's *t*-test $P < 0.001$, average root concentration 11.5 ± 1.5 µmol/g dry mass vs. average shoot concentration 6.1 ± 0.9 µmol/g dry mass) and were correlated at the individual plant level ($P < 0.001$ $R^2 = 0.17$). The extent of variation (over all cultivars) in glucosinolate concentrations was significantly larger in the roots than in the shoots (Levene's test $P<0.001$). Glucosinolate profiles also differed between roots and shoots (MCP $P = 0.002$ $F = 29.2$); all glucosinolates except glucoiberin and glucobrassicin were found in higher concentrations in the roots. Similar to what was observed for variation in glucosinolate concentrations, the extent of variation in glucosinolate profiles was larger in the roots than in the shoots (Levene's test $P<0.001$, variance in distances in the principal component analysis for root 0.385 and for shoot 0.047).

Degradation experiment

When analyzing the differences in degradation rates we found that overall, glucosinolates were degraded faster in non-sterilized than in sterilized soils (repeated measurements ANOVA $F = 4.1$ $P < 0.05$). When regarding the biosynthetic origin, aliphatic glucosinolates degraded faster than aromatic glucosinolates and aromatic glucosinolates degraded faster than indolyl glucosinolates (**Figure 4.4**, overall effect repeated measurement ANOVA $F = 23.1$ $P < 0.001$). Overall, however, we found no interaction between the biosynthetic origin of the glucosinolates and the soil sterilization treatment ($F = 2.7$ $P > 0.05$) indicating that the difference in the degradation rates between the different glucosinolate classes is not dependent on whether soil microbes are present or absent.

Comparison between plants grown in rhizotrons and plants grown in pots

In the first rhizotron experiment, the root glucosinolate profile of the *B. oleracea* cultivar Rivera differed from the two *R. sativus* cultivars (see section *comparison of glucosinolate profiles in roots and root exudates*). Similar differences were recorded in the pot experiment resulting in a non-significant interaction effect (see **Figure 4.3A** lines a and b; interaction effect MCP $F = 0.5$ $P > 0.5$). A similar trend can be observed when comparing the glucosinolate

profiles of plants used in the second rhizotron experiment with those in pots. Here the root glucosinolate profile of the *S. alba* cultivar Architect differed from the three *B. oleracea* cultivars both for plants grown in rhizotron (see above) and for those grown in pots. This resulted in a non-significant interaction effect (see **Figure 4.3B** line a and b, interaction effect MCP F =1.5 P >0.1).

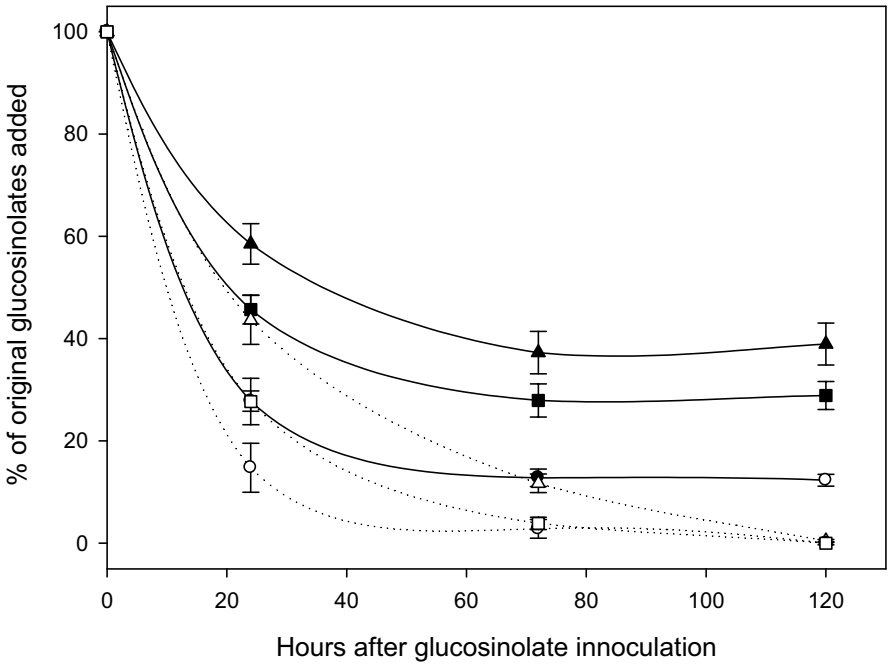

Figure 4.4 Degradation of glucosinolates from the soil ▲ Aliphatic glucosinolates ■ Aromatic glucosinolates ● Indolyl glucosinolates. Solid lines and black symbols: sterilized soil; dotted lines and white symbols: non-sterilized soil

Discussion

Two hypotheses were put forward to explain why belowground higher trophic level organisms and belowground organisms that are not directly associated with plant roots do not respond to intra- and inter-specific variation in root glucosinolate profiles. Our results provide support for the hypothesis that glucosinolate concentrations or profiles in the exudable fraction are not fully correlated with those found in the roots. Even though we found that total glucosinolates concentrations in the exudable fraction were correlated with those in the roots, the glucosinolate profiles in exudates differed significantly from those in the roots. We based this conclusion on the observation that inter-specific variation in root glucosinolate profiles was not reflected by a similar inter-specific variation in glucosinolate profiles in the exudable fraction. In the exudable fraction relatively more aliphatic glucosinolates were recorded than in the roots. The fact that inter-specific differences in root glucosinolate profiles are not reflected in the exudates might either be due to selective exudation (actively or passively) of glucosinolates, or due to differences in the rate of degradation of different glucosinolates. In the degradation test performed in this study we found that aromatic glucosinolates, which caused the inter-specific differences in root glucosinolates (gluconasturtiin and glucotropaeolin), degraded faster than aliphatic, but not than indolyl glucosinolates. The degradation test in this experiment was performed with purified glucosinolates without myrosinase, thus without hydrolyzing the glucosinolates. In biofumigation experiments, where myrosinase and glucosinolates are in direct contact, aromatic glucosinolates are the most stable (Gimsing and Kirkegaard 2006). Either way, with or without myrosinase the degradation of glucosinolates is depended on their biosynthetic origin.

Although glucosinolates degraded faster in non-sterilized soil, microbes are not responsible for selective degradation based on biosynthetic origin as we found no interaction effect between sterilization treatment and biosynthetic origin. This implies that differences in degradation rates between classes might be due to differences in chemical characteristics such as the immobilization by soil particles (Gimsing *et al.*, 2005; Gimsing *et al.*, 2009), or volatilization (Gimsing *et al.*, 2009). Either way, the glucosinolate profiles that are recorded in plant roots are not indicative for profiles in the exuded fraction.

Based on our data we could reject the second hypothesis that roots would show less variation in their glucosinolate concentrations or profiles than shoots. The extent of variation in glucosinolate concentrations and profiles was actually larger in roots than in shoots. Larger variation in glucosinolates belowground has been demonstrated previously and can be seen as a general trend for both wild and cultivated species (van Dam *et al.*, 2009).

To test the relevance of rhizotron experiments as predictors for plants grown under more commonly practiced growing conditions, we compared the glucosinolate profiles of the plants grown in rhizotrons to those in grown in pots. We found that profiles of roots from rhizotrons are representative for those growing under common experimental conditions. This, combined with the results from a previous experiment in which we demonstrated that plants growing in pots had root glucosinolate profiles similar to those in the field (chapter 2), lead to the conclusion that rhizotron experiments can indeed provide realistic predictions for inter- and intra-specific differences in glucosinolate profiles in the field.

In conclusion, root glucosinolates are exuded and can potentially influence the rhizosphere community. However, the glucosinolate composition of the exudate cannot be predicted from the glucosinolate profile of the root, even though total concentrations of glucosinolates in roots are correlated with those in the exudates. The inter-specific variation in glucosinolate profiles in the root was not accompanied by similar differences in the root exudates. Moreover, in soils with microorganisms, glucosinolates degrade rapidly. Both factors could explain why intra- or interspecific variation in root glucosinolate profiles is relevant for soil organisms directly associated with the roots, but less so for soil organisms that have no direct association with the roots or for higher trophic level soil organisms.

Chapter 5

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

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Abstract

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

Keywords; Plant-insect interactions, glucosinolates, tritrophic interactions, below-aboveground interaction, intra-specific variation, white cabbage, Brassicaceae, *Brevicoryne brassicae*, *Diaeretiella rapae*, Aphididae, parasitoid, Braconidae

Introduction

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

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

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

communities.

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

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

The objective of this study was therefore to examine whether plant intra-specific variation can affect the outcome of below-aboveground plant-mediated interactions up to the third trophic level. Making use of previous studies (Poelman *et al.*, 2009; Kos *et al.*, 2011a), we chose to examine below-aboveground interactions in two white cabbage cultivars that were known to differ amongst others in their root and shoot glucosinolate profiles and in their interactions with nematodes belowground and herbivores aboveground. We assessed whether soil treatments (nematodes or microorganisms) affect aboveground aphid populations of the specialist aphid *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae). The performance of this aphid is known to be

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

Materials and methods

Experimental setup

Seeds of two white cabbage cultivars – Rivera (breeder and seed source: Bejo Zaden, Warmenhuizen, The Netherlands) and Badger Shipper (breeder: University of Wisconsin, Madison, WI, USA; seed source: Centre of Genetic Resources, Wageningen, The Netherlands) – were germinated for 10 days in a growth cabinet, at L16(25 °C):D8(15 °C) hours. A total of 90 seedlings per cultivar were transferred to pots filled with 2 kg of a loamy, sandy mineral soil (0.13% N, 2.1% C, C/N = 16.7; particle size distribution: 3% <2 µm; 17% 2-63 µm; 80% >63 µm), which had been sieved (mesh size 5 mm) and gamma-sterilized at 25 kGrey (Isotron, Ede, The Netherlands). Pots were relocated to a greenhouse with L16(21 °C):D8(16 °C) hours, regulated by sodium lamps to maintain a minimum photoactive radiation of 225 µmoles m^{-2} s^{-1}. Plants were randomly divided over three treatments (30 replicates per cultivar per treatment). The treatments per cultivar consisted of (1) a control group of 30 plants without a soil inoculum, (2) 30 plants supplied with a nematode inoculum, and (3) 30 plants with only a microorganism inoculum.

To obtain a microorganism solution, 11 kg of the same unsterilized soil in which the plants were potted was suspended with 11 l of deionized water and incubated for 24 h at room temperature. The supernatant was passed over five sieves (1 × mesh size 75 µm, 3 × 45 µm, and 1 × 10 µm), which retained nematodes but allowed passing of most microorganisms. Aliquots of 70 ml were added to each pot of the microorganism treatment. Nematodes were extracted from the same soil as described above (but unsterilized) by filtering it over four sieves (1 × 75 µm, 3 × 45 µm) which retained the nematodes. The residue with the nematodes was purified by incubating for 24 h at room temperature on two milk filters suspended between clamps. This allowed nematodes to migrate into tap water; it was not possible to use deionized water because the osmotic potential could kill the nematodes. The resulting solution, inevitably containing microorganisms, was pooled and nematodes were counted in 10 subsamples to determine the density. To add 2000 nematodes per kg of soil, which is an average density for the soil type used in this experiment, 40 ml of the solution was required and added to the pots assigned for nematode treatment. We added 30 ml of tap water to supply the same amount of liquid as in the control and microorganism treatments. Control plants were supplied with 70 ml of demineralized water.

Aphid population growth and parasitoid performance

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

To monitor the effect on the third trophic level, 10 plants per treatment and cultivar were infested with 10 second instars that had been parasitized by the solitary endoparasitoid *D. rapae*. These aphids were reared on plants of the respective treatment, parasitized, and then again transferred to plants of the respective treatment. An aphid was considered parasitized

when the female parasitoid inserted her ovipositor in the aphid. From the day the first mummy (aphid remains containing a parasitoid pupa) appeared, plants were surveyed daily for new mummies. Mummies were then transferred into glass vials and from 7 a.m. until 11 p.m. checked every 2 h to record survival rate, egg-to-adult development time, adult dry mass, and sex ratio of the adult parasitoids. Since their collection from a Brussels sprouts field near Wageningen (the Netherlands) the parasitoids were reared in a climate room on *B. brassicae*.

Collecting plant and soil samples

From the start of the experiment until aphid infestation, the first and second leaves were photographed weekly and the size of the leaves was calculated by using a standard reference area, which was included in the photograph. From these photographs the leaf area was determined using WinFolia (Regent Instrument, Ottawa, ON, Canada). Based on these measurements, the relative growth rate was estimated as (ln area2 – ln area1)/(t2 – t1).

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

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

electrophoresis (DGGE).

After 29 days of aphid infestation, the remaining plants were harvested and the third leaf was, similar to the control plants, used for phloem collection as described above. Plant roots and shoots were separated, dried, and weighed.

Soil community analysis

To classify differences in microbial biodiversity, DNA was isolated from the rhizosphere soil using a PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the producer's instructions. Per treatment and cultivar we analysed four replicates. PCR-DGGE was performed as described in chapter 3. We analysed all treatments within a cultivar on the same gel, because it is generally difficult to compare gels.

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

Extraction and analysis of amino acids and glucosinolates

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

phloem solution. HPLC analyses were performed as described in chapter 2.

Statistical analysis

Nematode numbers were analyzed by two-way ANOVA. DGGE gel banding patterns were analyzed by distance based redundancy analysis (db-RDA) as described in chapter 3. However, data were visualized by correspondence analysis.

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

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

Results

Aphid population growth

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

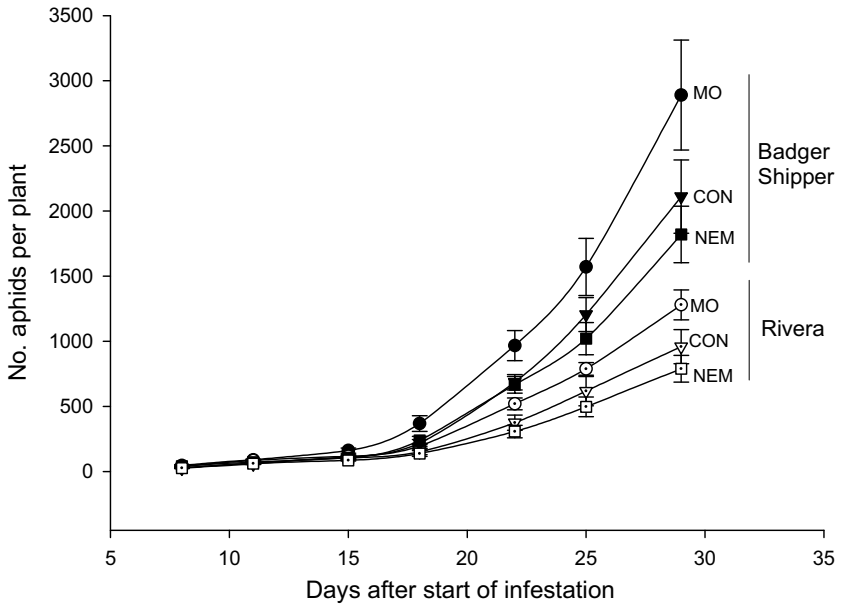

Figure 5.1 Aphid population development (mean ± SE number of aphids per plant). Aphids were reared on soils treated with microorganisms (○), untreated control soils (▽), or nematode-treated soils (□). White symbols represent aphids reared on Rivera, black symbols represent aphids reared on Badger Shipper.

Parasitoid performance

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

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

DGGE fingerprints and nematode numbers

For both Rivera and Badger Shipper, DGGE patterns of bacteria and fungi differed significantly between the treated soils on both the first and second axes of the db-RDA (MCP tests, Supplementary **Figure S5.1**). Before aphid exposure, total nematode abundances were significantly higher in nematode-treated soils, both in the bulk and in the rhizosphere soil, as was intended (**Table 5.1**). The number of phytophagous nematodes also differed between treatments and was several magnitudes higher in nematode-treated soils than in microorganism-treated and control soils (**Table 5.1**). The dominant nematodes belonged to the bacterivorous families Cephalobidae and Rhabditidae. Cultivar neither affected total nematode abundance, nor number of plant parasitic nematodes (**Table 5.1**). There were also no interaction effects between the cultivars and soil treatments on nematode abundances (**Table 5.1**).

Table 5.1 Average number of nematodes per treatment expressed per 100 g of dry soil or g dry root ± the standard deviation

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

Different letters within a column within a cultivar denote significant differences by Tukey post-hoc analysis

Plant chemistry

Glucosinolates

Glucosinolate concentrations in the phloem, leaves, and roots were not significantly affected by soil treatment (see Supplementary **Table S5.1**). Also the glucosinolate profiles were not significantly affected by soil treatment, neither in leaves (MCP: P>0.5; **Figure 5.2A**) or roots (MCP: P>0.1), nor in the phloem, before (MCP: P>0.1) or after aphid infestation (MCP: P>0.1).

Analogous to the aphid population growth data, differences in glucosinolate concentrations and profiles were much more pronounced between cultivars than between soil treatments. Phloem concentrations were significantly higher in Rivera than in Badger Shipper at the second sampling (Supplementary **Table S5.1**) and also the glucosinolate profiles in the phloem differed between the cultivars, both before and after aphid infestation (MCP: both P<0.01). Indolic glucosinolates, mainly 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin, dominated in the phloem of Badger Shipper, which had the highest aphid numbers, whereas in Rivera aliphatic glucosinolates, mainly gluconapin and sinigrin, dominated. No interaction between soil treatment and cultivar was recorded for glucosinolate profiles at either the initial (MCP: P>0.9) or the second (MCP: P>0.05) phloem sampling. In the roots and leaves, total glucosinolate concentrations did not differ between the cultivars, but their profiles did. Leaf glucosinolate profiles differed significantly between cultivars (MCP: P<0.01), with indolic glucosinolates

dominating in Badger Shipper (**Figure 5.2A**), similar to what was observed for the phloem glucosinolates. Root profiles also differed significantly between cultivars (MCP: P<0.01). Gluconasturtiin, which was not recorded in Badger Shipper, contributed to this difference. No interaction between soil treatment and cultivar was recorded for glucosinolate profiles in either leaves (MCP: P>0.9) or roots (MCP: P>0.05).

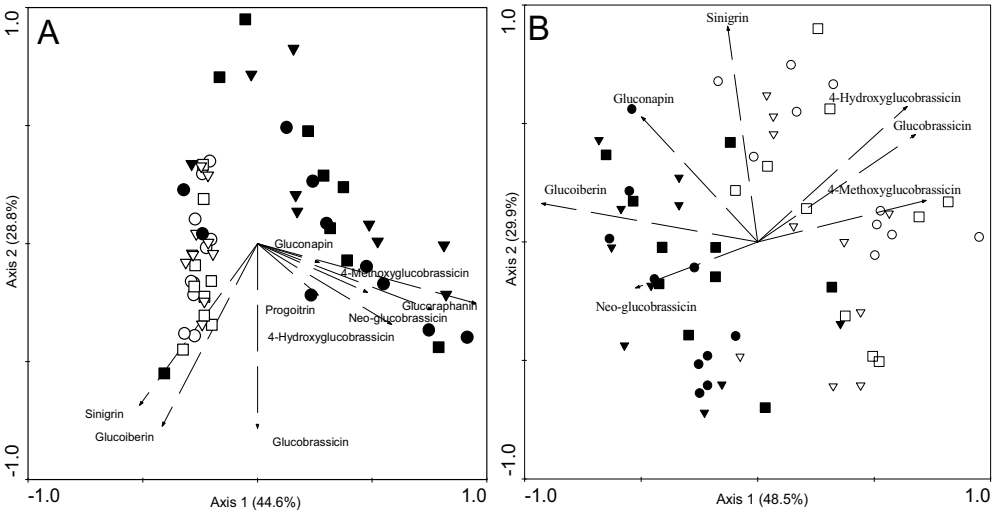

Figure 5.2 PCA plot for glucosinolate profiles of (A) leaf and (B) aphids; Plants or aphids raised on soils treated with micro-organisms (○), untreated control soils (▽), or nematode-treated soils (□). White symbols represent the cultivar Rivera, black symbols represent Badger Shipper. Arrows indicate different glucosinolates.

Amino acids. At the initial sampling, before aphid infestation, soil treatment did not affect phloem amino acid concentrations (P>0.8 Supplementary **Table S5.1**) or profiles (MCP: P>0.05). At the second sampling, *i.e.*, after aphid feeding, there was a significant soil treatment effect within Rivera. Compared to control plants, amino acid phloem concentrations were higher in this cultivar on microorganism-treated soils, which also had the highest aphid numbers, and on nematode-treated soils, which in contrast had the lowest aphid numbers (Supplementary **Table S5.1**). The differences in amino acid concentrations among treatments after aphid infestation were accompanied by significant differences in amino acid profiles (MCP: P<0.01).

Amino acid concentration in the phloem initially did not differ between the two cultivars. However, after aphid infestation, Rivera had considerably higher amino acid concentrations than Badger Shipper (Supplementary **Table S5.1**). The profiles differed significantly between

cultivars before aphid infestation (MCP: P<0.001). Rivera had higher levels of phenylalanine, whereas Badger Shipper had higher concentrations of all other amino acids. After aphid infestation the profiles also differed between the cultivars (MCP: P<0.001), with all amino acids dominating in Rivera.

Aphid chemistry

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

Plant biomass and leaf relative growth rate

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

Plant biomass did not significantly differ between the cultivars before aphid infestation. At the second sampling, after aphid infestation, both root and shoot mass differed significantly between the cultivars. Rivera had a higher root and shoot biomass than Badger Shipper. The relative growth rate of the first and second leaves between weeks 3 and 4 were significantly higher for Badger Shipper than for Rivera. There was no significant interaction between treatment and cultivar, indicating that cultivars responded similarly in relative growth rate and biomass to the treatments (Supplementary **Table S5.1**).

Discussion

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

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

The lack of differential effects between the cultivars in below

aboveground interactions might be due to the lack of an interaction between cultivar and soil treatment for nematode abundances. The cultivar Badger Shipper lacks the glucosinolate gluconasturtiin, which has been shown to negatively affect nematodes (Potter *et al.*, 2000). Yet, in our experiment, the nematode community under this cultivar was not different from that of the Rivera cultivar. This limited the scope for the two cultivars to differentially affect the aboveground community indirectly, through differential effects on their belowground nematode community. As different nematodes communities could result in differential upregulation of defensive compounds or mobilization of nutrients.

Soil inoculum effect

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

Effect of cultivar

It is generally known that intra-specific variation, also within the Brassicaceae, can affect aboveground herbivores and higher trophic level organisms (Crutsinger *et al.*, 2008; Kissen *et al.*, 2009; Newton *et al.*, 2009; Lankau, 2010). Our observation that Rivera sustained fewer aphids than Badger Shipper is consistent with a previous study (Broekgaarden, Poelman *et al.*, 2008). Aphid population growth generally is dependent on plant traits, such as leaf relative growth rate (Hughes & Bazzaz, 2001), and leaf or phloem concentrations of defensive compounds, such as glucosinolates (Kim, Lee *et al.*, 2008). Indeed the leaf relative growth rate did differ between cultivars. Moreover, the glucosinolates 4-methoxyglucobrassicin and glucobrassicin

dominated in the leaves and phloem of Badger Shipper, whereas gluconapin and sinigrin, which have been implicated to decrease aphid fitness (Cole, 1994), dominated in leaves and phloem of Rivera. Although phloem and leaf total glucosinolate levels were fluctuating (first higher in Badger shipper, later higher in Rivera), it might be the consistent differences in glucosinolate profiles that result in different aphid population growth rates. Replacing 4-methoxyglucobrassicin and glucobrassicin with gluconapin and sinigrin in *Arabidopsis thaliana* (L.) Heynh., analogous to the differences in glucosinolate profiles between Badger Shipper and Rivera in our study, reduced the population growth of *B. brassicae* on these plants (Kissen *et al.*, 2009). Therefore, the higher concentrations of gluconapin and sinigrin could have been one of the factors responsible for the reduced aphid numbers on Rivera.

Soil treatment did not result in different glucosinolate profiles among the aphids, but the cultivars on which they had been reared greatly affected their glucosinolate profiles. Intriguingly, the glucosinolate profiles in the aphids were completely different from those in the phloem: 4-methoxyglucobrassicin and glucobrassicin were recorded in higher concentrations in aphids on Rivera, whereas these dominated in Badger Shipper plants. The incongruence between glucosinolates in the plants and aphids may be due to the ability of *B. brassicae* to sequester glucosinolates, thus regulating glucosinolate composition (Bridges, Jones *et al.*, 2001; Kazana, Pope *et al.*, 2007). Hence, aphid glucosinolates are not necessarily directly correlated to phloem and leaf glucosinolate levels.

The different aphid glucosinolate profiles might explain why *D. rapae* performed better on aphids reared on Badger Shipper. On Badger Shipper, adult mass of *D. rapae* was higher and its development time was shorter, two parameters usually associated with beneficial host quality (Bukovinszky, van Veen *et al.*, 2008). However, on Rivera, female sex ratio was higher, also a parameter that is usually associated with better host quality (Tanaka, 2009). This incongruence may be related to temporal shifts in host quality. Adult mass and developmental time are dependent on the conditions after egg laying, so during the main part of the development of the host, whereas offspring sex ratio is determined by the female upon egg laying. Perhaps the effect of cultivar on host quality changes during the development of the host, therefore differentially affecting offspring sex ratio, determined by the female, and performance of the offspring.

In conclusion, our study has shown that aphid populations respond to both soil organisms and plant cultivar. However, below-aboveground

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

Supplementary material

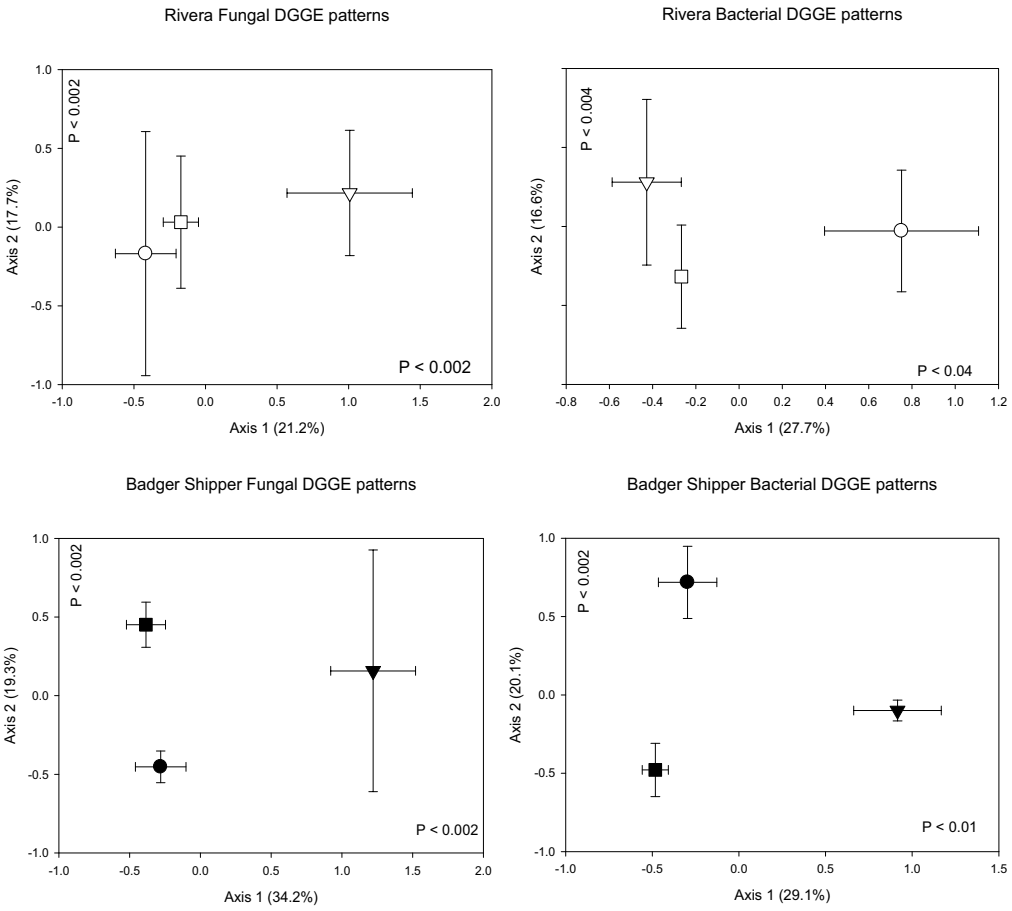

Figure S5.1 Correspondence analysis ordination diagram of denaturing gradient gel electrophoresis (DGGE) patterns of the bacterial and fungal communities. Points represent the mean of four samples with their 95% confidence interval. The P values on the first axis (lower right corner) and second axis (upper left corner) are determined by Monte Carlo permutation tests on the basis of distance-based redundancy analysis. This indicates that all treatments for both fungi and bacteria differ significantly from each other. White symbols represent samples from Rivera, black symbols represent Badger Shipper; ○ soils treated with micro-organisms, ▽ untreated control soils, □ nematode-treated soils.

Table S5.1 Average values of several plant characteristics, GLS = glucosinolate, RGR = relative growth rate, * denote overall significant differences (two-way ANOVA: P<0.05). Means within a line followed by different letters are significantly different (Tukey post-hoc analysis: P<0.05)

	Badger Shipper			Rivera			F		
	Microorganisms	Control	Nematodes	Microorganisms	Control	Nematodes	Cultivar	Treatment	Cultivar*treatment
Biomass root sampling 1 (g)	1.55 ab	1.89 ab	1.29 ab	1.880 ab	2.19 a	1.23 b	0.76	4.34*	0.34
Biomass shoot sampling 1 (g)	6.61 a	7.51 a	7.03 a	6.34 a	6.45 a	6.27 a	2.91	0.53	0.33
Biomass root sampling 2 (g)	3.97 ab	4.27 b	4.01 ab	5.93 ab	6.34 a	5.69 ab	7.96*	0.33	0.20
Biomass shoot sampling 2 (g)	15.04 a	17.54 ab	16.23 a	19.41 ab	21.91 b	21.58 b	20.97*	1.89	0.18
RGR (cm^2) first leaf, week 3-4	1.15 a	1.19 a	1.02 a	0.68 b	0.64 b	0.59 b	67.09*	3.10	0.45
RGR (cm^2) first leaf, week 4-5	2.37 a	2.13 a	2.20 a	1.99 a	2.15 a	1.98 a	4.49	0.36	1.60
RGR (cm^2) second leaf, week 3-4	0.56a	0.54 a	0.32 b	0.38 b	0.38 b	0.29 b	5.14*	3.04*	1.85
Total GLS concentration leaf (µmoles g^{-1} d.w.)	5.61 a	4.92 a	4.85 a	3.97 a	3.76 a	3.98 a	3.33	0.20	0.35
Total GLS concentration root (µmoles g^{-1} d.w.)	12.52 a	13.73 a	9.10 a	16.42 a	8.95 a	10.85 a	0.02	1.98	1.90
Total GLS concentration phloem before aphid (nmoles g^{-1} d.w.)	9.75 a	10.71 a	5.80 a	3.46 a	2.58 a	3.49 a	7.43*	0.23	0.46
Total GLS concentration phloem after aphid (nmoles g^{-1} d.w.)	15.68 ab	4.20 a	6.65 ab	16.33 ab	16.32 b	17.87 ab	5.59*	3.42	1.06
Total phloem amino acid concentration before aphid (µmoles g^{-1} d.w.)	13.84 a	11.40 a	8.04 a	9. 84 a	14.36 a	13.75 a	0.02	0.15	0.64
Total phloem amino acid concentration after aphid (µmoles g^{-1} d.w.)	1.63 ac	0.68 a	1.31 ac	5.32 b	1.89 ac	5.02 bc	57.32*	10.42*	1.51

Chapter 6

Effects of genetically modifying roots on rhizosphere processes and consequences for plant properties

Patrick Kabouw, Nicole M. van Dam, Wim H. van der Putten, and Arjen Biere

Abstract

Genetic modification of plants has become common practice. However, root-specific genetic modifications have only recently been advocated. Here, we review how root-specific modifications can have direct or indirect effects on aboveground plant properties. Direct mechanisms refer to processes occurring within the plant. Indirect mechanisms refer to effects that alter plant-rhizosphere interactions, which subsequently feed back to the plant. These plant-soil feedback mechanisms have been demonstrated both in natural systems and in crops. We discuss how direct and indirect effects could counteract improvements in plant properties for which the genetic modification was intended.

A literature survey revealed that rice is the most commonly studied crop species in the context of root genetic modification, predominantly in relation to stress tolerance. Phytoremediation, a process in which plants are used to clean up pollutants, is also often an objective when transforming roots. We use these two examples to review potential effects of root genetic modifications on shoots. Most root-specific genetic modifications lead to better plant performance only if the genes are specifically expressed in roots. Constitutive expression can even result in modified plants that perform worse than non-modified plants. Indirect effects have rarely been examined, but clearly genetic modification of roots can influence rhizosphere interactions, which in turn can affect shoot properties. Indeed field studies with root transformed plants often result in negative effects on shoots that are not recorded in laboratory studies. This might be due to the simplified environments that are used in laboratories which lack the full range of plant-rhizosphere interactions that are present in the field.

Keywords; Phytoremediation, hyperaccumulator, *Oryza sativa*, rice, plant-soil feedback, non-target soil organisms

Introduction

Roots and shoots are distinctly different in both form and function. Both organs have different gene expression patterns, metabolic profiles, and respond differently to environmental factors (Barabasz, Kramer *et al.*, 2010). Despite these differences, roots and shoots are intimately connected and mutually dependent on each other. Roots provide anchoring and supply nutrients and water; shoots in turn fix carbon and supply energy for growth and reproduction. Shoots and roots can interact via the transportation of plant metabolites, nutrients, and water through phloem and xylem (Uraguchi, Mori *et al.*, 2009). Changes in roots therefore can, but not necessarily have to, affect shoot processes. For example, when roots are exposed to stress, the biomass of the shoots may increase, as observed in response to some root biotic stresses (Wurst *et al.*, 2006) or decrease, as observed for a number of root abiotic stresses (Perez-Alfocea, Albacete *et al.*, 2008). Similar variation in shoot responses to root stress has been observed for other shoot characteristics such as the metabolite content of leaves or gene expression patterns (Jeong, Kim *et al.*, 2010). Potentially roots and shoots also respond differently to genetic modification, *i.e.* the introduction, over-expression, or silencing of a gene (Nap, Metz *et al.*, 2003). A novel modification that is introduced into a plant can thus be beneficial for roots and processes mediated by roots but detrimental for shoot properties, which may impede positive effects on plant production or yield.

The representation of genetically modified crops in agriculture is globally increasing. GM-crops are now grown in 29 countries, mainly owing to their enhanced productivity (James, 2010). However, genetic modifications in commercially available crops so far target specific aboveground properties, either by constitutive expression throughout the plant or by tissue-specific expression in the shoot (e.g. herbicide tolerance or insect resistance) (James, 2010). Modifications of root properties have only recently been recognized as an option to improve plant properties (Ghanem, Hichri *et al.*, 2011) and studies aiming at genetic modifications that target root properties are becoming increasingly popular (see **Figure S6.1**). However, genetic modifications that target root properties might interfere either positively or negatively with aboveground processes, similar to the pleiotropic effects that have been demonstrated for aboveground targeted modifications (Groot & Dicke, 2002). For example, the introduction of genes producing Bt-proteins,

one of the most commonly studied transformations, can either increase or decrease lignin concentrations in maize (Escher *et al.*, 2001; Saxena *et al.*, 2001), indicating how variable secondary effects of genetic modifications can be. Effects of genetic modifications on other traits than the targeted trait can arise either by direct or by indirect mechanisms.

Direct mechanisms are effects of genetic modifications that are caused by processes occurring within the plant. Indirect mechanisms refer to effects that alter the plant's biotic or abiotic interactions, which subsequently feed back to the plant. Direct effects can occur if the modified gene targets root processes without having a root tissue-specific expression. For example, a gene that is introduced to enhance resistance against soil pathogens may also be expressed in aerial parts (**Figure 6.1**, pathway *a*). These effects can be positive, for instance if the expression in aerial parts also provides resistance against aboveground pathogens, or negative, for instance if the gene product interferes with primary metabolism in aerial parts (Ge, Chen *et al.*, 2004). Direct effects can also occur if expression of the gene is restricted to the roots, but the product(s) that are formed by the modified gene are transported from the roots into aerial parts (**Figure 6.1**, pathway *b*). Like for pathway *a*, these direct effects may either be positive or negative.

Indirect effects of root-targeted genetic modifications can occur through feedback loops of the root modification with the rhizosphere. For instance, the modified gene or its product can positively or negatively affect the mobilization of plant nutrients (**Figure 6.1**, pathway *c*). Positive effects on mobilization of nutrients may result in positive effects aboveground, whereas negative effects on mobilization may result in self-inhibition. This means that when the plant grows, more modified gene product will be released and fewer nutrients will be mobilized. Another indirect pathway is that the modification can influence abundances of soil organisms (**Figure 6.1**, pathway *d*). This may result in positive effects if the modified gene unintentionally causes a reduction of belowground pathogens, for example if its product is toxic for plant pathogens. However, this indirect effect can also be negative, for instance if it reduces beneficial soil organisms that control plant pathogens or mobilize nutrients (Groot *et al.*, 2002).

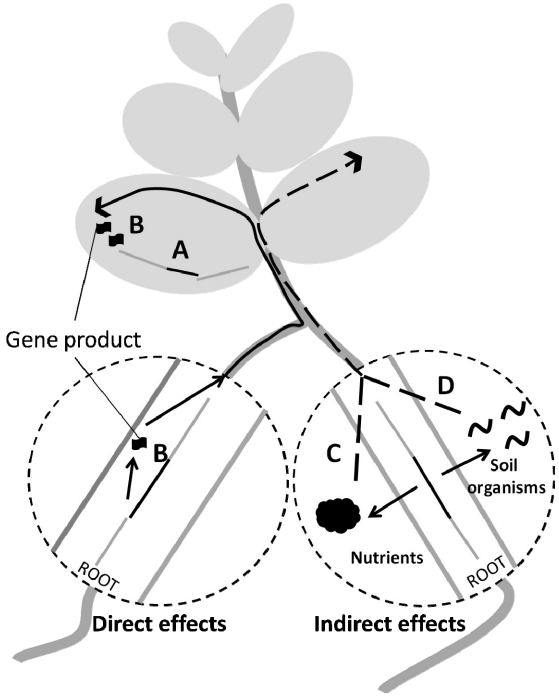

Figure 6.1. Illustration of how genetic modifications targeted at roots could potentially affect shoot processes and performance. Effects may be direct through expression of the modified gene in the shoot (pathway *a*) and through translocation of the modified gene product from the root to the shoot (pathway *b*), or indirect through rhizosphere interactions by increasing or decreasing nutrient mobilization (pathway *c*) and with soil beneficial and detrimental organisms (pathway *d*). Solid red lines are direct effects and dotted black lines are indirect effects.

Direct effects of root modifications on shoots have thus far received most attention, while indirect effects have frequently been ignored. However, these indirect effects, commonly called plant-soil feedback, can be just as important. Plant-soil feedback is thought to affect a range of biological processes in both wild and cultivated plants; including plant fitness, plant invasions, maintaining a diverse plant community, and succession of plants (for a review see Ehrenfeld *et al.*, 2005. Variation in plant-soil feedbacks can occur due to variation in even a single trait (Ehrenfeld *et al.*, 2005) and, therefore, genetic modification of a single trait could also influence plant-soil feedback.

If indirect effects of genetic modifications indeed enhance or reduce their intended benefit (e.g. an increase in productivity), this could explain the genotype by environment interactions that are often recorded when combining field and greenhouse trials with genetically modified plants (Anand, Zhou *et al.*, 2003; Birch, Griffiths *et al.*, 2007; Moon, Halfhill *et al.*, 2007). Owing to their simplified environments, greenhouse studies may involve less complex root-rhizosphere interactions than field studies (e.g. when nutrients are added in excess and the diversity of soil organisms is lower than in the field). Therefore, modified genes might have a beneficial effect in the greenhouse that is not counteracted by negative effects through altered plant-soil feedback. In field studies with more complex rhizosphere interactions (e.g. a diverse soil community and for example the presence of larger soil biota), negative effects of the genetic modification through altered interactions can potentially arise. However, indirect effects of genetic modifications on plant performance through plant-soil feedback have hardly been reported, as normally only the net effect of a modified gene on aboveground plant properties, such as productivity, is considered. Here, we will discuss evidence showing that modifications targeted at root processes can affect properties of aerial plant parts both directly and indirectly.

Most common root transformations and transformed plant species

As it has only recently been recognized that modification of root traits is a feasible option for improving plant properties (Ghanem *et al.*, 2011), no plants with modifications of root-specific processes have been released commercially yet (Macek, Kotrba *et al.*, 2008). Therefore, we will discuss root-specific modifications that have been reported most frequently in literature and take this as an indicator of future commercial release. To identify the most commonly transformed species and the nature of envisioned root-specific modifications, we evaluated the most common keywords in scientific studies when searching for the term “transgenic root” (see **Figure S6.2** for an explanation). The plant species that were most commonly recorded among these keywords are *Arabidopsis* (in 30% of the studies), tobacco (14%), rice (11%), maize (8%), *Medicago* (5%), potato, and tomato (both 4%). *Arabidopsis* and tobacco are both mainly used as model species. As rice seems one of the most commonly reported crop plants in relation to transformed roots, we will

use this species as a case study. The other commonly encountered keyword that we will discuss is "phytoremediation" (5% of the studies), meaning the use of plants to remove toxic compounds from polluted sites. Based on these keywords, we will discuss the possible direct and indirect effects of modifying rice roots and transforming plant roots for phytoremediation.

Case study one: root modifications in rice

Rice is the most important crop in the world as it provides food for roughly two billion people (Khush, 1997). However, it is also relatively prone to abiotic stresses such as drought and salt (Jeong *et al.*, 2010; Plett, Safwat *et al.*, 2010). Therefore, introducing tolerance to these stresses is the focus of many studies. Indeed, when examining the keywords that are frequently encountered in combination with "rice", we observed that "tolerance" and "resistance" to "drought" and "salt" are common transformations. In 11% and 6% of the studies the words "salt" or "drought" are encountered, respectively. Thus, we will focus on transformations increasing tolerance to salt and drought, although we will also mention other modifications. Tolerance to abiotic stress through genetic modification of roots can be achieved by several processes for example by physiological, cellular, and by molecular adaptation (Nguyen, Babu *et al.*, 1997; Mitra, 2001; Shinozaki & Yamaguchi-Shinozaki, 2007).

Direct effects of introducing root-specific genes

One of the ways to achieve enhanced drought tolerance in rice varieties is by genetic modification that results in plants producing more root biomass in deeper layers of the soil (Jeong *et al.*, 2010). However, this increased allocation of energy and resources to the roots might impair shoot growth (Price, Cairns *et al.*, 2002). Indeed, differences in root length between conventional rice varieties are correlated with shoot properties such as tiller numbers. Whether these correlations are negative or positive depends on the intensity of the drought (Babu, Nguyen *et al.*, 2003).

The introduction of genes intended to convey tolerance to drought or salt stress by promoting root development in deeper regions of the soil indeed has been shown to affect shoot traits. Over-expressing the *OsNAC10* gene in rice, a gene involved in auxin signaling, to promote lateral root development

with either a constitutive or a root-specific promoter resulted in increased tolerance to drought. However, compared to plants containing a constitutive promoter, plants with a root-specific promoter had a thicker root diameter, a more efficient photosynthesis (Redillas, Strasser *et al.*, 2011), and higher grain yield (Jeong e*t al.*, 2010) under drought conditions. This indicates that increased allocation of resources to root growth can have a net positive effect on yield. However, this effect is stronger when the transformed gene is expressed in a root-specific manner thus, when pathway *a* is avoided (**Figure 6.1**) (Ghanem *et al.*, 2011).

In another study, rice plants were made more tolerant to salt stress by introducing an *Arabidopsis* gene (*AtHKT1*) that is responsible for maintaining a low Na^+ translocation to the shoot by retrieval of Na^+ from the transpiration stream and sequestering Na^+ in roots (Plett *et al.*, 2010). However, increased tolerance was only achieved when the modified gene was specifically expressed in the root cortex. If the gene was constitutively expressed, plant growth was negatively affected. The constitutive expression of the gene might have increased Na^+ influx in all plant cells, counteracting the reduction in root to shoot Na^+ translocation (Moller, Gilliham *et al.*, 2009; Plett *et al.*, 2010) (**Figure 6.1**, pathway *a*). The direct negative effect of the modified gene when expressed in the shoots thus outweighs its benefit of the root process of increased sequestration.

Limited nitrogen uptake is also a major constraint in rice production. To increase rice biomass, an *alanine aminotransferase* gene from barley was introduced in rice. This gene is involved in nitrogen transportation and increased nitrogen uptake efficiency in the rice transgene (Shrawat, Carroll *et al.*, 2008). The introduction of the gene resulted in a bushier, finer, and more branched root system without increasing allocation of resources to the roots (Price *et al.*, 2002), as total root biomass was not increased (Shrawat *et al.*, 2008). Although the gene was expressed in a tissue-specific manner, mainly in the root epidermis, its protein product was also recovered in the shoot (**Figure 6.1**, pathway *b*). The modification had the desired effect, an increase in shoot biomass, but this was likely due to increased uptake of nutrients by the bushier and finer roots and not due to the presence of the modified gene product in the shoot (Shrawat *et al*., 2008).

Indirect effects of introducing root-specific genes

Conventional varieties of rice are sensitive to changes in the rhizosphere. This can subsequently result in negative or positive effects on plant properties. For example, the addition of soil protozoa (*Acanthamoeba castellanii*) to the rhizosphere of rice resulted in alteration of the bacterial community that in turn induced changes in root architecture (Kreuzer, Adamczyk *et al.*, 2006). The number of roots decreased whereas the average length of the roots increased. This subsequently affected the shoot, which contained more nitrogen when exposed to protozoa (**Figure 6.1**, pathway *d*) demonstrating that changes in the rhizosphere community composition can result in altered aboveground properties. Based on this we expect that if a genetic modification changes the abundance or diversity of soil organisms, as was mimicked by the addition of protozoa, this could give rise to either positive or negative plant-soil feedbacks. Several modifications that have been introduced in rice specifically target soil pathogens. These modifications could potentially affect non-target soil organisms and subsequently shoots, but since they have mainly been studied in laboratory trials in the absence of non-target organisms, such effects have not yet been documented.

Genetically modified rice plants expressing chitinase genes in their roots showed enhanced control of fungal pathogens (Xu, Zhu *et al.*, 1996), but also negatively affected non-target organisms such as non-pathogenic soil bacteria and mycorrhizal fungi (Yang, Yuan *et al.*, 2002). Mycorrhizal colonization of rice is an important determinant for yield (Solaiman & Hirata, 1997) and (partial) loss of mycorrhizal associations can lead to increased root growth but decreased shoot growth (Kothari, Marschner *et al.*, 1990; Secilia & Bagyaraj, 1994). In another study, genetically modified rice plants expressing a proteinase inhibitor to control nematodes (*Meloidogyne incognita*) experienced 50% lower abundances of this nematode in laboratory experiments (Vain, Worland *et al.*, 1998). In a field study, potato plants transformed with the same gene affected not only nematode abundances but also non-target microbes. In this field study, the modification had no or a negative net effect on biomass production (root, tuber, and shoot) compared to non-transformed potato plants, depending on the season (Cowgill, Bardgett *et al.*, 2002). However, it is difficult to attribute the reduced plant productivity to the effects of the modified gene on soil microbes (**Figure 6.1**, pathway *d*). In both rice and potato, the modified gene was constitutively expressed (**Figure 6.1**, pathway *a*), which could also have counteracted the initial beneficial result of lower plant pathogenic nematode numbers via direct effects on the

shoot.

Genetic modifications to enhance drought or salt tollerance in rice may have similar effects on non-target organisms or on nutrient mobilization as described above for chitinase and proteinase inhibitor genes. However, to date, no studies have addressed this, so that we can only speculate about the possible effects. For instance, Jeong *et al.* (2010) showed that modification of drought tolerance in rice by over-expressing the *OsNAC10* gene in the roots resulted in pleiotropic effects on other genes that have a key function in nutrient mobilization. It was recorded that heavy metal transporter genes in the roots, and to a lesser extent in the shoots, were upregulated as a result of the root-targeted modification. This is in line with the observed connection between metal accumulation and drought tolerance in rice (Yang, Wu *et al.*, 2009). Consequently, modifying roots to enhance drought tolerance might increase metal uptake (**Figure 6.1**, pathway *c*), which could result in higher metal concentrations in aboveground plant parts. This can lead to both positive and negative effects in aboveground plant parts, as metals represent essential nutrients but can also lead to oxidative stress.

Case study two: phytoremediation.

Many sites in the world are contaminated by pollutants, such as heavy metals, herbicides, and other chemicals. A common procedure to clean these sites is to excavate them and treat or isolate the soil, which is costly and laborious. As an alternative, plants can be used to accumulate environmental pollutants. This method, called phytoremediation, is becoming increasingly popular (Eapen & D'Souza, 2005). Plants take up the pollutants via their roots, followed by translocation and subsequent sequestration in shoots. However, most plants that accumulate large quantities of pollutants are relatively small (Pomponi, Censi *et al.*, 2006; Kawahigashi, 2009). One of the ways to increase the overall efficiency and the rate at which contaminated sites can be cleaned is by modifying pollutant-accumulating plant species to increase their size. Alternatively, large plant species that are normally unable to accumulate pollutants can be genetically modified to increase their tolerance and ability to take up large quantities of pollutants. The popularity and expected future potential of these modifications is reflected in the large number of reviews published on this subject (Kramer & Chardonnens, 2001; Clemens, Palmgren

et al., 2002; Gisbert, Ros *et al.*, 2003; Eapen *et al.*, 2005; Kramer, 2005; James & Strand, 2009; Kawahigashi, 2009; Kotrba, Najmanova *et al.*, 2009; Kramer, 2010; Rascio & Navari-Izzo, 2011). However, from these reviews and our own literature survey it is clear that there is not one specific plant species that is frequently transformed, nor is there a specific pollutant at which genetic transformations are targeted. Modifications targeted at roots to increase phytoremediation potential primarily focus on enhancing root tolerance to stress caused by the accumulated pollutants (Arshad, Saleem *et al.*, 2007), increasing root biomass to accumulate more pollutants, (Eapen, Suseelan *et al.*, 2003), and excreting proteins that mobilize or degrade pollutants in the plants' rhizosphere (Wang, Li *et al.*, 2004).

Direct effects of introducing root-specific genes

Modifying plants to tolerate higher levels of pollutants such as heavy metals is one of the mechanisms to increase phytoremediation potential. To increase the tolerance to nickel, a common environmental pollutant, a bacterial gene that produces 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) was introduced in tomato and canola, plants that are normally unable to accumulate pollutants. ACCd hydrolyses ACC, which is a precursor of ethylene. Normally exposure to nickel enhances ethylene production which initiates senescence and chlorosis in the shoots and decreases root growth (Arshad *et al.*, 2007). The production of ACCd by transgenic plants should result in decreased stress responses in their roots by decreasing ethylene levels. Tomato and canola plants with root-specific expression of ACCd were significantly better at maintaining their biomass and increasing nickel concentrations in shoots compared to non-transformed plants and plants that constitutively expressed the gene (Grichko, Filby *et al.*, 2000; Stearns, Shah *et al.*, 2005). Actually, constitutive expression of the ACCd gene resulted in a direct negative effect on shoot biomass when exposed to nickel, similar to what was observed when non-transformed plants were exposed to nickel (Grichko *et al.*, 2000; Stearns *et al.*, 2005). Therefore, the expression of a root-targeted genetic modification in the shoot in this case resulted in a direct negative effect on the shoot (**Figure 6.1**, pathway *a*).

Field tests with the canola lines used by Stearns *et al.* (2005) revealed that these plants were indeed able to maintain a higher biomass than non-transformed plants (Farwell, Vesely *et al.*, 2006). The nickel content, however, was similar to that of non-transformed plants (Farwell *et al.*, 2006). The

intended benefit of the transformation, which was recorded in the laboratory, might have been reduced in the field as a result of indirect effects (**Figure 6.1**, pathway *c* or *d*). As no plants under a constitutive promoter were used no direct effects could not be assessed in the field study by Farwell *et al.* (2006).

Indirect effects of introducing root-specific genes

Currently root modifications for phytoremediation are mainly evaluated in laboratory studies. It is known that in field situations, transgenic plants that constitutively express genes involved in enhancement of phytoremediation can accumulate larger quantities of pollutants than non-transgenic plants, while maintaining their biomass (Banuelos, Terry *et al.*, 2005). For root transformed plants, such effects have not yet been observed in the field (Farwell *et al*., 2006). However given the potential of positive indirect effects, root modifications might well have the desired effect on biomass and accumulation potential when grown on contaminated sites. For instance, field studies with conventional metal-accumulating plants have shown that when specific microorganism communities are present the phytoremediation of metals and biomass increases (Audet & Charest, 2007; Ma, Prasad *et al.*, 2011). Interestingly, growing metal-accumulating plants specifically promotes these microorganism communities (Audet *et al.*, 2007; Pongrac *et al.*, 2008). Thus, there is the potential for a positive feedback, as modifying plants for increased metal uptake can additionally result in favorable soil communities, which in turn increase the amount of accumulated metals (**Figure 6.1**, pathway *d*).

In a laboratory study by Stearns *et al*. (2005), a root-specific promoter was used to increase metal uptake by canola by introducing ACCd. Canola and many other plants that are genetically transformed for enhanced phytoremediation of heavy metals belong to the Brassicaceae (Eapen *et al.*, 2005), which are characterized by a class of secondary metabolites, the glucosinolates. Glucosinolates are involved in plant defense (Mithen, 2001) and are considered to be at least partly responsible for the lack of an association of Brassicaceae with mycorrhizal fungi (Vierheilig *et al.*, 2000; Pongrac *et al.*, 2008). Previously, it has been shown that increased metal concentrations lead to decreased glucosinolate concentrations (Poschenrieder, Tolra *et al.*, 2006; Pongrac *et al.*, 2008). This trade-off enables Brassicaceous plants (e.g. *Thlaspi*) to form associations with mycorrhizal fungi when accumulating high metal concentrations (Pongrac *et al*., 2008). Genetic modifications that increase metal uptake can therefore potentially have an

indirect positive effect on the plant's fitness through enhanced association with mycorrhizal fungi, provided that these are able to increase plant nutrient uptake (**Figure 6.1**, pathway *d*).

Pollutants such as phenolic compounds, can also be removed from contaminated sites *ex planta.* This is achieved by the release of metabolites from the roots that degrade the pollutants in the rhizosphere. For example, transformed *Arabidopsis* and tobacco that excrete laccase degrade and tolerate higher concentrations of phenolics (Wang *et al.*, 2004; Sonoki, Kajita *et al.*, 2005), whereas wild-type *Arabidopsis* plants suffer from chlorosis under these conditions (Wang *et al*., 2004). However, the excretion of laccase could result in increased susceptibility to plant pathogens. Several (fungal) plant pathogens naturally excrete laccase (Mayer & Staples, 2002; Strong & Claus, 2011) to enhance their ability to infect host plants as laccase can play a role in the detoxification of plant metabolites involved in defense (Mayer *et al.*, 2002). The excretion of laccase by genetically modified plants could thus result in increased fungal infection by enhancing the detoxification of the plant's own defenses. On the other hand, contradicting this idea, it has been observed that some plant species naturally produce laccase in response to fungal pathogens. The functional significance of this is not yet entirely clear (Mayer *et al.*, 2002). Possibly, fungal signals responsible for host finding might be disrupted. Either way, the production of laccase is likely to influence the infection rate of plant pathogenic fungi (**Figure 6.1**, pathway *d*). Such changes in the infection rate might result in indirect effects in the aerial parts.

Examining indirect effects of root-targeted modifications

Several methods are available to examine the direct effects of the modification of a gene. It can be assessed where and to what extent the modified gene is expressed (e.g. by Q-PCR or GFP enhancer trap lines), where its gene product is located (only in the root or also in the shoot), and whether other genes are up- or down regulated due to the modification (e.g. by using microarrays) (Jeong *et al.*, 2010; Plett *et al.*, 2010; Ghanem *et al.*, 2011). Although time consuming and expensive, these analyses are widely applied and relatively easy compared to the evaluation of indirect effects and their mechanisms.

Indirect effects of root-targeted modifications are difficult to establish and even harder to quantify. As a consequence, they are rarely evaluated, as

also appears from our literature search. Nevertheless, there are tools available for accurate assessment of these processes. A commonly used approach in soil science when assessing treatment effects is to closely monitor sensitive indicator species (e.g. mycorrhizal fungi) and/or processes (e.g. nutrient turnover) in the rhizosphere. This same approach can be used to examine how the genetic modification of plants affects feedback mechanisms in their rhizosphere. A selection of sensitive indicator non-target species and/or processes can be made that have the potential to feed back to the plant when their abundances or performance are affected by the genetic modification (e.g. mycorrhizal fungi). These species and/or processes can be monitored subsequently (Bruinsma, Kowalchuk *et al.*, 2003). This approach is relatively straightforward, but carries the risk of missing feedback mechanisms when organisms and/or processes that cause them are initially quantified as trivial and thus not selected and monitored. Another approach is a large-scale evaluation of soil organisms and processes. With the substantial improvements in the (molecular) toolboxes (Simon & Daniel, 2011) the use of these high-throughput methods is becoming increasingly more feasible. There are several approaches available for the large scale evaluation of soil organisms and processes. For instance, next-generation sequencing techniques allow the screening of both the taxonomic (by considering species specific genes) and functional diversity (by including information on functional genes) of soil organisms (Simon *et al.*, 2011).

Whatever approach is chosen, the evaluation of effects on rhizosphere processes and species composition can improve our understanding of the effects of modified genes. It can also improve the quality of the modified plants in field situations, as it allows for fine-tuning genetic modifications in order to reduce negative and promote positive effects. Additionally, the evaluation of these interactions and the use of modern molecular techniques are frequently a requirement for risk assessments (Bruinsma *et al.*, 2003) and should be initiated at some stage during application for approval of genetically modified crops.

Conclusions

We have argued that besides potential direct effects of root-targeted modifications there might also be indirect effects through altered plant-rhizosphere interactions that feedback to the plant, affecting its growth and reproduction. In general, genetic modifications that are intended to affect root processes result in enhanced plant performance when the modified gene is specifically expressed in the root compared to when it is expressed in both roots and shoots. This was highlighted by several studies where constitutive expression led to modified plants that actually performed worse than non-transformed plants. With respect to indirect effects, it has been demonstrated both in natural systems and for conventional crops that single traits are able to affect plant rhizosphere interactions and that these altered interactions can have a profound impact on plant performance. Although rarely examined, these effects can also be expected for root-targeted genetic modifications that have the potential to generate a plant-soil feedback effect. These effects could occur if the modification alters the uptake of valuable and/or harmful elements or via changed abundances of pathogenic or beneficial soil organisms. Our review shows that for several modifications there could indeed be rhizosphere interactions reducing the beneficial effects intended by the modification. With the advent of new molecular techniques, high throughput assessment of soil processes is possible and more feasible. Therefore, we recommend that both direct and indirect effects should be evaluated in early stages of greenhouse and field tests of plants with root-targeted genetic modifications. This can enhance the mechanistic understanding of the modification and increase the quality of the modified plant.

Supplementary material

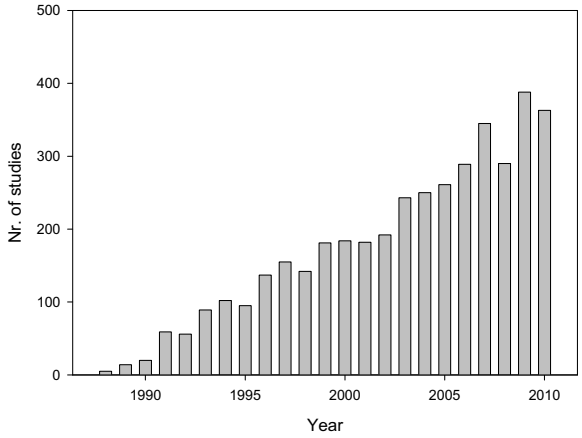

Figure S6.1: Number of studies encountered when searching for the term 'transgenic root"

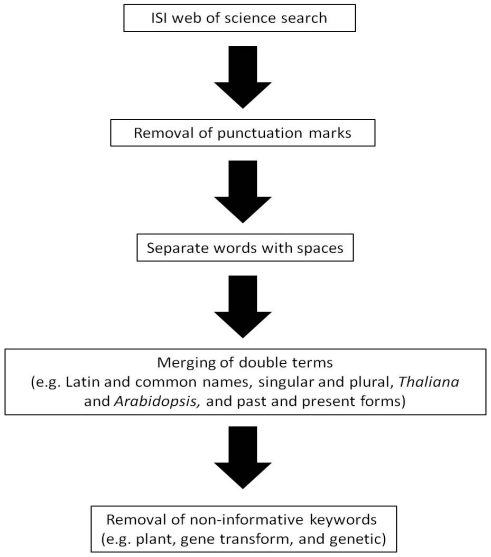

Figure S6.2: Flow chart of how the list of keywords obtained from ISI Web of Science by searching for the term "transgenic root" was processed.

Flow chart of how the list of keywords obtained from ISI Web of Science by searching for the term "transgenic root" was processed. All papers from ISI Web of Science were evaluated that were recorded when searching for the term "transgenic root" while constraining time between January 1st, 2007 and April 15th 2011. The top 5 of the most commonly encountered keywords in those studies were expression (168x), *Arabidopsis-thaliana* (131x), plants (127x), *Arabidopsis* (82x), and gene (75x), very general terms and terms referring to similar plant species or root processes. The list of keywords had to be processed to remove duplication of keywords and conflicting keywords. Therefore, we removed hyphens and dashes, so that for example *Arabidopsis-thaliana* and *Arabidopsis thaliana* would not be seen as different terms. Then terms that were divided by a space were separated (so *Arabidopsis* and *thaliana* became two separate terms). Subsequently, duplicated terms were merged (e.g. *Arabidopsis* encompassed for example *Arabidopsis* and *thaliana*).

Chapter 7

Discussion & Synthesis

In this thesis I studied effects of intra-specific differences in plant traits on soil organisms, the mechanisms behind these effects, and the consequences of these effects in a below-aboveground context. For this I used white cabbage as a model system, as these plants exhibit large intra-specific variation in morphology, physiology, gene expression, and metabolites (Sturtevant, 1887; Broekgaarden *et al.*, 2007a; Poelman *et al.*, 2009; Kos *et al.*, 2011a; Kos *et al.*, 2011b). Based on the results obtained from these studies I have speculated how varieties that are newly created through the use of genetic modification could affect these same processes and I will discuss how it can be tested, using multivariate statistics, whether these effects fall within or outside the baseline of effects observed for conventional varieties.

I took an approach of combining greenhouse and field studies. I screened a large number of varieties in the greenhouse for intra-specific variation, and tested field effects on soil organisms with a subset of the varieties, representing the most extreme ones with respect to their metabolic profiles. This approach allowed me to examine if effects on soil organisms recorded in the field and in the greenhouse are correlated. After establishing effects of intra-specific variation on soil organisms in the field I examined the mechanisms behind these effects and their consequences for below-aboveground interactions under more controlled experimental conditions in the greenhouse. The main results obtained in this thesis are summarized in **Figure 7.1**. These results include the effects of plant intra-specific variation on plant-associated and non-target organisms, both belowground and aboveground, as well as interactions between below- and aboveground organisms.

In this chapter I will discuss the approach that I chose and the main results presented in this thesis. I will give recommendations for how to evaluate effects of genetically modified plants. In addition, I show how to statistical assess whether effects of genetically modified plants fall within or outside the baseline variation.

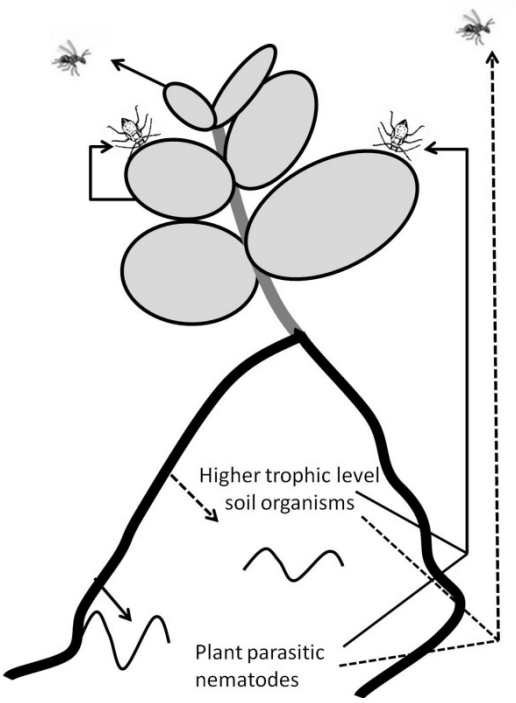

Figure 7.1: Schematic overview of the interactions involving White Cabbages studied in this thesis. Solid lines indicate significant effects, dotted lines indicate non-significant effects. Intra-specific variation can affect both belowground (chapter 3) and aboveground organisms (chapter 4). However, while intra-specific variation can affect non-target organisms aboveground (chapter 4), belowground non-target organisms are not affected (chapter 3). Belowground organisms also influence aboveground organisms that are directly associated with the plant (chapter 5), however not higher trophic levels (chapter 5).

General discussion

In chapter 2 I show that the cultivars that were used for the initial screening differed significantly in their glucosinolate profiles, even though their total glucosinolate concentrations were not different. Intra-specific variation in glucosinolate profiles in the roots was larger and more consistent between environments (greenhouse and field studies) than that of the shoots.

Both below- and aboveground, intra-specific variation in glucosinolates resulted in effects on organisms that are directly associated with the plant. Belowground, plant parasitic nematode numbers were affected (chapter 2) while aboveground, aphid population growth was affected

(chapter 4). However, I found little evidence that belowground organisms that are not directly associated to the plant were affected by glucosinolates, whereas aboveground also organisms that are not directly associated to the plant were influenced by intra-specific variation in glucosinolates (parasitoid development, chapter 4). The effect on aphids is in accordance with previous studies reporting that aboveground higher trophic levels can be influenced by intra-specific variation in glucosinolates or their breakdown products (Bukovinszky, Gols *et al.*, 2005; Kos *et al.*, 2011a; Kos *et al.*, 2011b). Non-target organisms belowground are also known to be influenced by glucosinolates although this has only been shown in laboratory studies (Jensen *et al.*, 2010). However, apart from effects on plant-parasitic nematodes, I found no effects on other organisms in the soil (chapter 3). Negative effects of cultivars on the root lesion nematode *Pratylenchus sp*. were associated with variation in one specific glucosinolate, gluconasturtiin. Previously, Potter *et al.* (1998; 2000) observed that increasing the gluconasturtiin concentrations in plants reduces the abundance of *Pratylenchus*. Individual glucosinolates aboveground have also been found to affect other organisms. For example, plants that had high glucoiberin concentrations harbored fewer herbivores (Poelman *et al.*, 2009; Kos *et al.*, 2011a). The results presented in this thesis and these previously published studies confirm that it is not the total concentration of glucosinolates, but rather the amount of individual glucosinolates, *i.e.* the profiles, are responsible for effects on plant associated organisms.

The observation that belowground organisms that are not directly associated to the root were not affected by the intra-specific variation in glucosinolates (chapter 3) can be explained by the results presented in chapter 5. In chapter 5 I tested whether the glucosinolates recorded in plant roots are also found in root exudates. Indeed this seemed to be the case: glucosinolates were recorded in the root exudates although in relatively low concentrations. The total concentrations that were recorded in the root exudates were positively correlated with the concentrations in the roots. However, the profiles of the glucosinolates in roots and exudates were not correlated. As a consequence, the large intra- and inter-specific variation in root glucosinolate profiles was not reflected in similar differences in the root exudates. For soil organisms the differences in glucosinolate profiles are more important than the total concentrations (see chapter 3). The absence of a correlation between glucosinolate profiles in roots and root exudates might thus explain why belowground organisms that are not directly associated to the plant do not respond to intra-specific variation in root glucosinolate

profiles. The most likely explanation for the lack of correlation between the profiles of the roots and root exudates are differences in degradation rates between glucosinolate types. As shown in chapter 5 and previously (Gimsing *et al.*, 2005; Gimsing *et al.*, 2009), the degradation of glucosinolates in the soil is dependent on their molecular structure. The differences in degradation rates are probably caused by differences in chemical characteristics of the glucosinolates that either result in different rates of immobilization (Gimsing *et al.*, 2005; Gimsing *et al.*, 2009) or in different rates of volatilization (Gimsing *et al.*, 2009).

I found that belowground organisms are capable of affecting aboveground organisms through alteration of their shared host plant (chapter 5). The presence of microorganisms promoted aphid populations, whereas nematodes tended to decrease aphid populations. One possible explanation for the positive effects of microbes on the aphids is that microorganisms mobilize nutrients that increase plant quality and subsequently aphid populations. Nematodes could have reduced microorganism abundances, as the majority of the nematodes in the soil were bacterivorous. This could result in less mobilized nutrients and lower plant quality and aphid numbers. The effect of soil organisms was, however, limited to aphids and did not extend to the third trophic level, the parasitoids. This is in contrast to previous studies that recorded effects of belowground organisms on higher trophic levels aboveground (Bezemer *et al.*, 2005; Soler, Bezemer *et al.*, 2005; Soler *et al.*, 2007). The contrast between these studies and my results might be explained by differences in the soil communities (e.g. bacterivorous vs. plant parasitic nematodes), the plant species, or aphid and parasitoid species that were used.

The main question with regard to below-aboveground interactions was if intra-specific variation can alter the outcome of these interactions. Although this has been previously hypothesized for both inter- and intra-specific variation (Sell *et al.*, 1990; Bezemer *et al.*, 2005; Wurst *et al.*, 2008) I did not find this for the intra-specific variation between the two cabbage cultivars. The two most divergent white cabbages varieties, Rivera and Badger Shipper (chapters 2 & 3 and see (Kos *et al.*, 2011a; Kos *et al.*, 2011b) showed similar patterns in their below-aboveground interactions. Indeed both varieties responded similarly to the belowground communities with respect to concentrations and profiles of primary (amino acids and sugars) and secondary (glucosinolates) metabolites. In contrast to my hypothesis and the results from chapter 3, no variety-specific soil community was recorded, as I found no differences in the nematode or microorganism communities

between these two cultivars. This might have limited the potential of these two divergent varieties to alter below-aboveground interactions. Previously, it was recorded that below-aboveground interactions were similar on two different grass species and two different oats cultivars (Sell *et al.*, 1990; Bezemer *et al.*, 2005). Thus, in my studies below-aboveground interactions seem to be consistent among varieties, even though the interactions themselves depend on the soil organisms involved.

Predictive value of greenhouse studies for field effects

The use of both greenhouse and field studies allowed me to test how indicative greenhouse studies are for field effects and how consistent intra-specific variation is between these environments. In greenhouse studies I found intra-specific variation in the glucosinolate profiles of white cabbage cultivars in both roots and shoots (chapters 2 & 4). The intra-specific variation that was recorded in the roots was consistent between greenhouse and field. However for the shoots the intra-specific variation that was recorded in the greenhouse was not reflected by the intra-specific variation in the field. This larger plasticity of glucosinolate profiles in shoots compared to roots might be due to different biotic selection pressures experienced by roots and shoots during evolutionary history (van Dam *et al.*, 2009). It has been speculated that roots are more consistently exposed to herbivory and therefore require a constantly high level of defense expression, whereas shoots may experience more variable biotic stresses, thus making it profitable to respond by inducible defenses, instead of allocating limited resources to constitutive defenses (Kaplan *et al.*, 2008; van Dam *et al.*, 2009). Indeed aerial parts are known to increase glucosinolate concentrations in response to stresses such as herbivory (Gols, Bukovinszky *et al.*, 2008; van Dam *et al.*, 2008), in contrast to the roots (chapter 2). This means that for shoots, greenhouse studies, which generally represent a low-stress environment, have only a limited predictive value for assessing the intra-specific variation in glucosinolate profiles in the field. By contrast, root glucosinolate profiles from greenhouse studies appear to be good indicators for glucosinolate profiles in the field.

However, root glucosinolate profiles have no predictive value for the glucosinolate profiles of the root exudates. The intra- and inter-specific differences that were recorded in the roots of plants grown in the greenhouse

could not be recorded in the root exudates. In chapter 4 I demonstrate that soil microorganisms can accelerate the degradation of glucosinolates. Additionally, the organic matter percentage of the soil affects the degradation rate; sandy soils have a lower degradation rate than clay soils (Gimsing, Sorensen *et al.*, 2006; Laegdsmand *et al.*, 2007; Gimsing *et al.*, 2009). As agricultural field soils contain more organic matter and more microorganisms than the sandy soils used in the greenhouse study in this thesis it is likely that in the field soil exuded glucosinolates are catabolized even faster. Probably, in the field, glucosinolate profiles of the roots have even less predictive value for the profiles in the exudates.

In contrast to the consistency of root glucosinolate profiles, effects of glucosinolates on soil organisms are not consistent between the greenhouse and field studies. Plant-parasitic nematodes (*Pratylenchus sp.*) were affected by intra-specific variation in glucosinolate profiles in the field (chapter 3), but not in the greenhouse (chapter 4). This might be due to the relatively short time span of the greenhouse experiment (8 weeks vs. 19 weeks of the field experiment) in which plant parasitic nematodes may have had little time for reproduction. Another reason might be the differences in the soils that were used. In the greenhouse, a more sandy soil was used that might not have been a very suitable habitat for plant parasitic nematodes (Kable & Mai, 1968; Lazarovits, Hawke *et al.*, 1991), explaining their overall lower abundances. If I would not have performed the field experiment, I would have missed the effects on the plant parasitic nematodes.

As discussed earlier, aboveground I recorded that both lower and higher trophic level organisms were affected by the intra-specific variation in white cabbage. Aphid population growth on Badger Shipper was approximately two-fold higher than on Rivera and also parasitoid performance was overall better on Badger Shipper (Chapter 5). I only evaluated effects on aboveground organisms in the greenhouse. However, another study evaluated the effects of both Rivera and Badger Shipper on the same aphid and parasitoid species in the field (Kos *et al.*, 2011a). In the field, aphid population sizes were also consistently larger on Badger Shipper than on Rivera, but the magnitude of the difference between these two cultivars ranged from four-fold to fourteen-fold (Kos *et al.*, 2011a). For the parasitoids, individual performance, as measured in this study, was not recorded in the field, so that for this species no direct comparison between greenhouse and field results can be made. Nevertheless, parasitoid numbers were also higher on Badger Shipper than Rivera (Kos *et al.*, 2011a), indicating better

performance on the former cultivar, similar to what I recorded in my greenhouse study.

In conclusion, intra-specific variation in the glucosinolate profiles of the roots recorded in a greenhouse can be a good indicator for intra-specific variation in glucosinolates in the field. However, this appears to be not the case for the shoots; greenhouse studies are not indicative of intra-specific variation in shoot glucosinolate profiles in the field (**Table 7.1**). The opposite holds true for the effects of intra-specific variation on organisms. Greenhouse effects of intra-specific variation in shoots are more indicative of the observed field effects than those observed for soil organisms (**Table 7.1**).

Table 7.1 Comparison between recorded effects in greenhouse and field experiments. Asterisks indicate that recorded field and greenhouse effects were correlated, n.s. means not significant. For the effects on aboveground organisms, no direct comparison can be made between the field and greenhouse.

Greenhouse studies		Field study
Intra-specific variation in root glucosinolates (chapter 2,4 & 5)	*	Intra-specific variation in root glucosinolates (chapter 2)
Intra-specific variation in shoot glucosinolates (chapter 3 & 4)	n. s.	Intra-specific variation in shoot glucosinolates (chapter 2)
Intra-specific variation does not affect soil organisms (chapter 4)	n. s.	Intra-specific variation affects soil organisms (chapter 3)
Intra-specific variation does affect aboveground organisms (chapter 4)		Intra-specific variation does affect aboveground organisms (see ref text.)

Recommendations for evaluating effects of genetically modified plants

Genetic modification of plants can potentially affect the diversity or abundance of non-target organisms (Crawley, Hails *et al.*, 1993). The evaluation of these effects is a requirement during risk assessment procedures for new transgenic varieties. Commonly this evaluation is done by comparing two varieties, the transformed plant and its isogenic line (*i.e.* the parental cultivar that was modified). When comparing the transformed plant to its isogenic line, significant effects on biodiversity have been recorded (Losey, Rayor *et al.*, 1999; Andreote, Mendes *et al.*, 2008). However, even if differences

in effects between a transgenic plant and its isogenic line are statistically significant, the magnitude of the difference may be small when comparing differences between the effect of the isogenic line and that of another variety (*i.e.* the full baseline). For instance, when effects of transgenic plants on biodiversity were compared with that of a set of randomly selected varieties, it was observed that the effects of transgenic plants do not exceed those of this baseline (Cortet, Griffiths *et al.*, 2007; Griffiths, Heckmann *et al.*, 2007). In this thesis I have shown that a range of varieties differing in their glucosinolate profiles can significantly affect both belowground and aboveground organisms, demonstrating that conventional varieties already exhibit a large range of effects. Thus to assess effects of a genetically modified variety in relation to effects already displayed by conventionally bred varieties, it may be more effective and scientifically sound to first evaluate effects within the range of conventional varieties before comparing the transformed plant with this baseline. In the statistics box I will give some recommendations of how to evaluate whether a specific variety such as a genetically modified plant falls within or outside this baseline.

My results also show that the effects of genetically modified plants on soil organisms should be evaluated in the greenhouse as well as in the field. Greenhouse studies, even of conventional varieties that differ in glucosinolate profiles, have only limited predictive value for effects in the field. Soils in greenhouses can be simplified environments that may lack the full array of plant-rhizosphere interactions that are present in the field (see also chapter 6). Greenhouse studies can, however, be used to select varieties for assessing baseline effects. For instance, for root glucosinolate profiles, the selection of this set of cultivars was done in the greenhouse and I found that profiles are consistent between greenhouse and field experiments. For the shoots, greenhouse screening of glucosinolate profiles did not yield results that could be used to characterize variation in these metabolites in the field. To what extent this holds for other plant traits, and the effects of genetic modification of these traits, remains to be investigated.

Based on the literature available, I suggest that there may be interactions between the products of the genetic modification and rhizosphere processes, which could feed back to the plant (negative or positive transgenic-plant soil feedback). For example, a modification may have a negative effect on the mobilization of nutrients or the abundance of beneficial soil organisms and this can result in reduced plant growth. These complex interactions are

Statistical recommendations for examining intra-specific variation in relation to base line variation

Introduction

Genetic modification of plants can result in varieties that have effects on non-target organisms that fall outside the range of variation in effects observed for conventional varieties (the baseline). Multivariate statistics (MVS) based on ordinations are useful tools to identify varieties that represent extremes for a given set of measured traits, and to establish whether specific varieties fall within or outside the baseline variation. I used MVS to assess whether specific varieties differed from a range of other varieties in their glucosinolate profiles (chapters 2 & 4) and to test for differences in their effects on soil organism as a consequence of intra-specific variation in glucosinolate profiles (chapters 3 & 5). In general, MVS are useful tools to classify samples, *i.e.* to visualize differences between samples for which multiple variables have been recorded. However, there are a number of challenges that can be encountered when using MVS. In particular, it is important to not ignore important variation and to correctly assess whether observed differences are statistically significant, *i.e.* whether effects of transgenic plants fall within or outside the baseline.

How not to ignore important variation

Publications using MVS frequently show and interpret only the first two components. However, third and higher components of ordination diagrams might still contain meaningful information, for example intra-specific variation among varieties. Interpretation of higher components might also reveal a variety with an extreme phenotype that was not visible when assessing the first two components only. Including this variety would allow selection of the correct baseline. On the other hand, analysis of too many components will result in analyzing meaningless information (i.e. the random error). For correct interpretation of MVS it is important to determine how many of the components produced by the MVS model should be analyzed. To do so, it is necessary to have an objective criterion for making a correct decision about the number of components to be retained for correct interpretation. There are several methods to evaluate how many components should be included in MVS models. In this thesis two methods were used, namely scree plots and Horn's parallel analysis (both used in chapter 2). Although there are also other methods (e.g. cross validation, broken stick, or Kaiser method), Horn's parallel analysis was found to be one of the best to evaluate the significance of components (Zwick & Velicer, 1986; Lautenschlager, 1989; Watkins, 2000; Hayton, Allen *et al.*, 2004). This method randomly generates components based on random data with similar dimensions as the real data set before it compares these randomly generated components to the original components. Each component is evaluated sequentially. Only when a specific component (e.g. the first) explains more variation than the randomly created components of the same order (also the first) should it be used for the interpretation of the data. The use of Horn's parallel analysis can increase the accuracy of MVS interpretation and thus lead a better determination of effects of intra-specific variation

How to determine if observed differences are within or outside of the baseline

The second challenge after all interesting components have been identified is that a researcher is interested in knowing whether the observed differences, e.g. between a genetically modified variety and the baseline, are statistically significant. Frequently this is tested by using the loadings of the samples (*i.e.* the position of the sample in the ordination space) in more "traditional" (univariate) statistics after an ordination. However, many MVS transform the original values, thereby altering the likelihood of finding significant differences, thus leading to compromised accuracy. Obviously, data transformation is not unique for MVS, as it is also common practice in traditional analyses such as ANOVA to fulfill assumptions of these models.

However, in ANOVA transformations are intentional, as they are actively performed by the researcher prior to the analysis, while this is not necessarily the case in MVS where transformations can be inherent in the methods. For instance, in non-metric dimensional scaling (nMDS) the sample arrangement in ordination space is not representing the real distances, but only the rank order. Basically it is irrelevant whether samples differ by a magnitude of 10-fold or 100-fold; the representation in the nMDS ordination will remain the same (Legendre & Legendre, 1998; Lepš & Šmilauer, 2003). In ecological studies, however, the real magnitude of differences is of relevance and not only the ranking. Would one perform "traditional" statistical tests on the metrics derived from these transformed ordinations, one would be interpreting transformed information and might miss effects caused by intra-specific variation or find that variation is significant whereas in fact it is not. For example, samples in an ordination may seem more alike than the samples in an ecological sense are. When evaluating whether effects of genetically modified plants fall within or outside the baseline variation, the use of transformed data might lead to misinterpretation of the effect of the genetically modified plant relative to the baseline. Therefore, analyses should preserve the original scaling of the calculations underlying a MVS analysis (as done in chapter 2).

An alternative to complicated and inaccurate "traditional" analyses following MVS are for example Monte Carlo permutation tests. The advantage of the Monte Carlo permutation test is that they are performed on the raw data underlying the ordination diagram. This results in a more accurate analysis of the underlying trends (Anderson & Legendre, 1999; Legendre & Anderson, 1999).

Conclusions

MVS are useful tools for examining intra-specific variation and the effects thereof, for example to visualize and test whether effects of genetically modified plants fall within or outside the baseline variation. However, the accuracy of MVS can be improved by selecting the correct number of components for interpretation, based on objective methods like Horn's parallel analysis. For determining whether specific varieties differ from other varieties or the baseline, Monte Carlo permutation tests are recommended.

not always present to their full extent in greenhouse studies as they are in the field, because soil in the greenhouse may lack some, for example large, soil organisms, it commonly has additional nutrients supplied, and is kept under controlled conditions. Field soils, on the other hand, contain a diverse array of soil organisms, could be limited in their nutrient availability, and have more fluctuating environmental conditions. This might explain why beneficial effects of plant properties that have been altered by genetic modification are recorded in the greenhouse, but not in the field where complex negative rhizosphere interactions may neutralize the positive effect of the modified genes. Therefore, testing the effect of genetic modifications in the greenhouse as well as in the field should be common practice, as has been recommended before (Kos, van Loon *et al.*, 2009).

Conclusions

Intra-specific variation in glucosinolate profiles of white cabbage can result in differences in the abundances of plant associated organisms both below- and

aboveground. However, in contrast to aboveground organisms, organisms belowground not directly associated to the plant were not affected by intra-specific variation in glucosinolate profiles in this study. This was likely due to a lack of intra-specific variation in the glucosinolate profiles in the root exudates. I also recorded that belowground organisms can affect aboveground organisms, but that in my study these interactions were not dependent on intra-specific variation in glucosinolates. This was likely due to the lack of a variety-specific effect on soil organisms that prevented differential feedback effects on the cultivars regarding plant quality and subsequent differential effects on aboveground multitrophic interactions. This does not preclude that variety-specific soil feedback mechanisms can affect processes in aerial parts. I have outlined this for transgenic plants in which the modification of a gene can result in both negative and positive feedback mechanisms through interactions with the rhizosphere.

To evaluate ecological effects of genetically modified plants I recommend the use of a baseline approach. This is the screening of a large number of varieties in the greenhouse before selecting a smaller subset of these varieties, using appropriate statistical tools like multivariate statistics, and assessing effects of this subset in the field. Greenhouse studies can be used to select varieties that represent the extremes with respect to the traits or effect of interest. These extremes can be used in field tests to evaluate effects on for example soil organisms and thus to assess the baseline effects. In greenhouse tests, effects of genetically modified plants can be evaluated in relation to this baseline to see if effects of genetically modified plants fall within or outside this baseline. However, when evaluating effects of genetically modified plants, greenhouse studies cannot be taken as proxy for their ecological effects in the field as they lack complex plant-soil interactions that are present to their full extent in the field. Greenhouse studies should therefore rather be seen as a first step in the whole evaluation process of evaluating the effects of genetically modified plants than as a final judgment.

Samenvatting

Plant intra-specifieke variatie, dwz variatie in eigenschappen binnen een plantensoort, kan organismen beïnvloeden die direct afhankelijk zijn van planten. Deze effecten kunnen bijvoorbeeld worden veroorzaakt door verschillen in plantenkwaliteit die het gevolg zijn van variatie in nutriënten gehalten of verdedigingsmechanismen. Plant intra-specifieke variatie kan ook niet-doelorganismen zoals hogere trofische niveaus en organismen die niet direct interacteren met aan de plant, beïnvloeden. Deze effecten kunnen optreden door verschillen in de kwaliteit van de planteneters die als gastheer of prooi dienen voor hogere trofische niveaus of als gevolg van verschillen in de aantrekkelijkheid van de plant voor parsitoïden en predatoren. Intra-specifieke variatie komt van nature voor in wilde planten. Bovendien heeft kunstmatige selectie op planteneigenschappen die wenselijk zijn vanuit de landbouw praktijk geresulteerd in nieuwe variëteiten, wat nog eens extra bijdraagt aan de intra-specifieke variatie in cultuurgewassen.

Naast natuurlijke variatie en variatie tussen landbouw rassen die door de mens gebruikt worden, kan genetische modificatie van planten leiden tot variëteiten met nieuwe eigenschappen zoals verhoogde productiviteit of resistentie tegen ziekten en plagen. Deze eigenschappen kunnen mogelijk ook niet-doelorganismen beïnvloeden. In dit proefschrift staat de vraag centraal of effecten van gemodificeerde planten op niet-doelorganismen binnen of buiten de bandbreedte van effecten vallen die waargenomen worden bij conventioneel veredelde variëteiten. Om deze vraag te beantwoorden is het echter eerst noodzakelijk om te bepalen wat de effecten zijn van conventionele variëteiten, de zogenaamde 'baseline'. In dit proefschrift heb ik baseline-effecten van conventionele wittekool variëteiten op bodem-organismen onderzocht, de mechanismen achter deze effecten, en de gevolgen voor de interacties tussen onder- en bovengrondse organismen. De effecten die deze conventionele variëteiten hebben, kunnen dan dienen als basis voor het evalueren van de effecten van genetische modificaties.

In dit proefschrift ben ik begonnen met het karakteriseren van intra-specifieke variatie in wittekool variëteiten door het meten van glucosinolaatconcentraties en -profielen. Glucosinolaten is een verzamelnaam voor een groep van circa 120 secundaire plantenstoffen die voornamelijk aanwezig zijn in kruisbloemigen. Glucosinolaten kunnen zowel bovengrondse

als ondergrondse organismen beïnvloeden. Uit mijn onderzoek blijkt dat glucosinolaatprofielen in zowel de wortels als de bladeren van wittekool variëteiten intra-specifieke variatie tonen. De variatie in glucosinolaatprofielen in de wortels van vier wittekool variëteiten heb ik verder gebruikt om de effecten hiervan op verschillende bodem-organismen te bestuderen. Ik ontdekte dat plantenparasitaire nematoden worden beïnvloed door de verschillen in de glucosinolaatprofielen van de wortels, terwijl ik geen effecten kon vaststellen op de niet-doelorganismen. Deze laatste waarneming zou mogelijk verklaard kunnen worden door het feit dat de glucosinolaatprofielen van wortelexudaten veel minder intra-specifieke variatie vertoonden dan de wortels. De totale glucosinolaatconcentratie in de wortels was positief gecorreleerd met die in de wortelexudaten. Echter, de glucosinolaatprofielen van wortels en wortelexudaten waren verschillend en het aantal individuele glucosinolaten was lager in de wortelexudaten dan in de wortels. Uit mijn experimenten blijkt dat dit mogelijk verklaard kan worden door de verschillen in degradatie snelheden van de individuele glucosinolaten in de bodem.

Door het toevoegen van verschillende groepen van bodem-organismen aan gesteriliseerde grond onderzocht ik hoe bodem-organismen via hun gedeelde waardplant bovengrondse organismen kunnen beïnvloeden. Voor dit experiment gebruikte ik twee wittekool variëteiten die uiteenliepen wat betreft hun glucosinolaatprofielen en hun effect op de bodem-organismen. Micro-organismen die werden toegevoegd aan de bodem bevorderden de groei van bladluispopulaties, terwijl de toevoeging van nematoden de groei van de bladluispopulatie juist remde. De richting van het effect van de toegevoegde bodem-organismen was overeenkomstig in beide variëteiten, hetgeen aangeeft dat de uitkomst van onder-bovengrondse interacties niet werd beïnvloed door intra-specifieke variatie in de plant die als intermediair diende voor de interacties tussen de onder- en bovengrondse organismen.

Genetische modificatie van plantenwortels zou indirect invloed kunnen hebben op de groei van deze gemodificeerde planten, bijvoorbeeld als de modificatie invloed heeft op de bodem-organismen, die vervolgens weer plantprocessen beïnvloeden. Deze mechanismen staan ook wel bekend als de zogenaamde bodem-plant terugkoppelingseffecten. Ik heb deze mogelijkheid onderzocht door het reviewen van recente literatuur over planten met genetische gemodificaties gericht op wortelprocessen. Ik concentreerde me hierbij op twee transformaties, namelijk rijstplanten, die zijn aangepast aan droogte en planten getransformeerd om vervuilende stoffen op te nemen uit

de bodem en deze te tolereren. Uit mijn literatuurstudie blijkt dat er inderdaad terugkoppelingseffecten tussen bodem-organismen en planten kunnen optreden. Dit zou kunnen resulteren in zowel positieve als negatieve terugkoppelingeffecten van de genetische modificatie op plantprocessen, zoals de bedoelde hogere opbrengst.

Voordat men kan beoordelen of effecten van genetisch gemodificeerde planten binnen of buiten de bandbreedte van conventionele variëteiten vallen, is een goede kennis van deze effecten bij conventionele variëteiten vereist. In dit proefschrift heb ik hiervoor, in ieder geval voor wittekool, de basis gelegd, vooral wat betreft interacties tussen de plant en bodemorganimsen en tot op zekere hoogte ook voor de interacties tussen combinaties van ondergrondse en bovengrondse organismen. Conventionele wittekool variëteiten verschillen in hun effecten op de bodem-organismen en deze effecten kunnen mogelijk resulteren in gewijzigde onder-bovengrondse interacties, zoals werd gesimuleerd door de toevoeging van specifieke groepen van bodem-organismen aan de grond. Mijn onderzoek kan dienen als een aanpak voor het bepalen van ‘baseline’ effecten van genetisch gemodificeerde wittekool en andere gewassen.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Patrick Kabouw
Date: 1 February 2012
Group: Terestrial Ecology, NIOO-KNAW & Nematology, Wagenigen University & Research centre

1) Start-up phase	date
► **First presentation of your project**	
Ecological effects of plant resistance traits on belowground non-target organism in different genotypes of Brassica and Arabidopsis	Dec, 2007
► **Writing or rewriting a project proposal**	
Ecological effects of plant resistance traits on belowground non-target organism in different genotypes of Brassica and Arabidopsis	Dec 2007
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
► **EPS theme symposia**	
Theme 3 symposium 'Metabolism and Adaptation', University of Amsterdam	Feb 18, 2009
Theme 2 symposium 'Interaction between Plants and Biotic Agents, Utrecht University	Jan 15, 2010
► **NWO Lunteren days and other National Platforms**	
NIOO days	Jun 2008
NIOO days	Jun 2009
NERN days	Feb 2008, 2009, 2010 & 2011
► **Seminars (series), workshops and symposia**	
Workshop plant insect interaction	Oct 26, 2007
Food webs in aquatic and terrestial ecosystems	Nov 11, 2009
Seminars at the NIOO-KNAW (once a month)	2007-2011
EPS & PE&RC symposia	2007-2011
► **Seminar plus**	
► **International symposia and congresses**	
Glucosinolate congress, Elsinore, Denmark	May 24-27, 2009
BES Meeting The integrative role of plant secondary metabolites in ecological systems 2010	Apr 12-14, 2010
IBC, Melbourne, Australia	Jul 23-30, 2011
► **Presentations**	
Poster presentation multitrophic interaction workshop Göttingen	Mar 25-26, 2010
Presentation at the Agraroekologie (Göttingen)	Apr 2010
Presentation IBC 2011 Melbourne	Jul 23-30, 2011
► **IAB interview**	Dec, 2009
► **Excursions**	
Subtotal Scientific Exposure	*14,2 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Identification course nematodes	Feb 04-15, 2008
Winter School Ecology of Plant Volatile Organice Compunds	Nov 11-15, 2008
CANOCO course, multivariate analysis of ecological data	Jan 20-31, 2009
PhD course Root-soil microbe Interactions	Jan 24-28, 2011
► **Journal club**	
PhD discussion group	2007-2011
► **Individual research training**	
DGGE training	start 2008
HPLC training	end 2007
Subtotal In-Depth Studies	*16,0 credits**

4) Personal development	date
► **Skill training courses**	
Training workshop data management for scientific data, Vlaams Instituut voor de Zee	Jul 02-04, 2008
Writing and presenting scientific papers	Dec 15-18, 2009
► **Organisation of PhD students day, course or conference**	
Organisation of NERN session 2011 (ecology regarding genetically modified plants)	2010-2011
► **Membership of Board, Committee or PhD council**	
EPS educational committee	2008-2011
Subtotal Personal Development	*5,0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.7**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	

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