Arriving at the right time:

A temporal perspective on above-belowground herbivore interactions

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"时间就像海绵里的水，只要愿意挤，总还是有的。"

- 鲁迅

"Time is a drug. Too much of it kills you."

- Terry Pratchett

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Chapter 1

General introduction

Aboveground-belowground interactions

Plants encompass two compartments, aboveground shoots functioning in photosynthesis and belowground roots functioning in nutrient and water acquisition. Both plant compartments are associated with a variety of organisms. Many studies have shown that organisms colonizing one plant compartment can induce biochemical and physiological responses of plants thus influencing performance, reproduction, population development and community composition of organisms within the other compartment (Bardgett and Wardle 2003; Wardle et al. 2004; van der Putten et al. 2009; Bardgett and Wardle 2010). The research theme "above-belowground interactions" is to examine how aboveground organisms can interact with belowground organisms via their shared host plants, and *vice versa* (van der Putten et al. 2001).

The aboveground parts of plants are colonized by various organisms including herbivores such as herbivorous insects and grazing mammals. These aboveground herbivores can damage plant aerial tissues, induce specific plant responses and affect plant fitness. For example, they can induce plant defense that protect plants from further damage of the current attacks or the damage from subsequent herbivores (Karban and Baldwin 1997). In particular plants can further benefit if the defenses are systemic and also inhibit damage of belowground herbivores (Box 1.1, Heil and Bostock 2002).

Different soil biota have distinctly different functions and they can both directly or indirectly influence plant growth. Accordingly, belowground organisms can differ greatly in how they induce responses in a plant and mediate the performance and fecundity of aboveground organisms. For instance, in many ecological systems belowground herbivores can systemically induce defenses in plant foliage and negatively influence aboveground herbivores (Bezemer 2003 et. al; Soler et al. 2005; Wurst and van der Putten 2007; Kaplan et al. 2008b). Similarly, arbuscular mycorrhizal fungi (AMF) tend to prime plants for defenses and confer an enhanced level of defense against generalist chewing aboveground herbivores that

subsequently arrive (Pozo and Azcón-Aguilar 2007). Whereas above-belowground interactions have been extensively studied in recent years (van Dam and Heil 2011) the majority of these studies only investigated these interactions at a single time point, and the temporal dynamics of aboveground-belowground interactions have been largely neglected (but see Bardgett et al. 2005; Erb et al. 2011).

Box 1.1 Local defense vs. systemic defense

Plants can be exposed to aboveground and belowground antagonists such as viruses, bacteria, fungi, nematodes, insects or mammals. Upon attack by these organisms plant can be induced and express defense against them (Green and Ryan 1972). These induced defenses can be limited to the site of damage termed "*Local defense*" or also expressed in the distant plant parts that are not damaged termed "*Systemic defense*" (Davies and Schuster 1981).

In recent years, many studies have shown that damage of plant tissues by herbivores can induce systemic defense in undamaged tissues and even across plant compartments (van der Putten et al. 2001). Thus, organisms separately colonizing plant aboveground and belowground tissues can be linked by these systemic defenses, a research topic called "above-belowground interactions".

Plant-herbivore interactions

In nature, plants are frequently colonized and attacked by a variety of insect herbivores above and below ground. Some insects remove plant tissues, feed from phloem or xylem or form specialized galls in plant organs (Marquis 1984; Maron 1998). Other herbivores can bore or mine plant reproductive organs and consume plant structures such as pollen and seeds. These insect herbivores can reduce plant survival and fitness throughout a plant's lifetime. To adapt to these challenges plants have evolved a variety of defense strategies to survive and maintain fitness (Karban and Baldwin 1997).

Plant defense is defined as plant traits or responses that reduce damage by herbivore attacks and contribute to maintaining plant fitness (Karban and Baldwin 1997). Since plants are immobile organisms and cannot escape from enemies, they defend themselves by producing physical barriers, repellents, toxins, and digestibility reducers to directly ward off herbivores (Chen et al. 2008; Mithöfer and Boland 2012), or by enhancing the attraction and performance of the natural enemies of their herbivores by emitting volatiles to recruit predators or parasitoids (Kessler and Baldwin 2001) as well as by providing food or shelter to them (van Rijn et al. 2002). The former way of plant defense is called "direct defense" and the latter one is termed "indirect defense" (Vet and Dicke 1992). Both defenses can benefit plants via reducing herbivory damage or mitigating the negative impacts of their damage (Box 1.2).

Box 1.2 Direct vs. indirect plant defenses

Direct defenses

Direct defense includes plant traits that can by themselves reduce a plant's susceptibility to herbivore attack (Kessler and Baldwin 2002). Multiple direct defenses are used by plants, such as physical barriers and chemical toxins. Physical barriers include physical structures like trichomes (Traw and Feeny 2008), spines (Gowda and Palo 2003) and thorns (Milewski et al. 1991) that plants can use in significantly reducing the consumption rate of herbivores (Hanley et al. 2007; Tian et al. 2012; Eaton and Karban 2014). Alternatively, plants can also defend themselves by synthesizing toxic chemicals to deter herbivore feeding and avoid potential further attacks. These chemicals are abundant in plants, including alkaloids (Ohnmeiss and Baldwin 2000), glycosinolates (Hopkins et al. 2009), terpenoids (Langenheim 1994), and phenolics (Boeckler et al. 2011) and can comprise up to one third of plant dry biomass (Obst 1998). Although these defensive structures or chemicals are intrinsic traits of plants to a certain level (constitutive defense) they can also be induced to a higher level (induced defense) when plants are exposed to a certain level of biotic or abiotic stresses (Kutyniok and Müller 2013).

Indirect defenses

Indirect defenses specifically indicate plant traits that serve to recruit and to enhance the performance of natural enemies of the herbivores. Plant traits involved in indirect defenses include the production of volatile organic compounds (VOCs), extrafloral nectaries (EFNs), food bodies (FBs, plant produced structures containing liquids and proteins, Heil et al. 1997) and structures used as refuges and nesting spaces, such as domatia (Heil 2008). All these products can attract a variety of predatory and parasitizing animals such as predatory mites (Dicke 1986) and parasitoids (Turlings et al. 1990) as well as parasitic nematodes (Rasmann et al. 2005). Similar to direct defense, the synthesis of these compounds can also be induced upon damage by herbivores. For example, the VOCs can be induced both upon aerial (Paré and Tumlinson et al. 1999) and upon root damage (Rasmann et al. 2005).

Root herbivory effects on shoot herbivore growth

Root insects Impacts of root insects on the performance of shoot herbivores depend on both the insect type and plant species examined. Several early studies have shown that root herbivory by insects can benefit the growth of foliar aphids since root herbivory causes damage to fine roots which results in a reduction of water and nutrient uptake of the plant, known as "Stress Response Theory" (Gange and Brown 1989; Masters et al. 1993). The deficiency of water could in turn lead to the elevation of concentrations of amino acids and carbohydrates in the foliage and ultimately enhance performance of foliar aphids. However, a later study reported higher aphid performance in foliage of root-damaged plants but without any increase of soluble N in root herbivore-damaged plants (Johnson et al. 2009). The failure of several studies linking aphid performance to water or nutrient stress of the host plant has further challenged this hypothesis (Koricheva et al. 1998; Huberty and Denno 2004).

Negative effects of root herbivory on leaf chewers are more commonly reported than positive effects (Bezemer et al. 2004; van Dam et al. 2003; van Dam et al. 2005; Soler et al. 2005). These studies usually witnessed a systemic

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Box 1.3 Constitutive vs. induced plant defense

Constitutive defense

Optimal defense theory suggests that plant defenses are costly and should be mainly invested in plant parts that are of greatest value to plant fitness (Zangerl and Bazzaz 1992). Plant tissues that are more likely to be attacked by herbivores should possess higher levels of constitutive defenses since it is too risky to only rely on inducible defenses which are not expressed immediately after attacks (Zangerl and Rutledge 1996). On the other hand, if plants would allocate constitutive defenses to plant tissues that are rarely attacked by enemies, it would reduce plant fitness at the expense of resources that could be invested in other functions like growth or production. This is also important within an above-belowground context given that exposure of roots or shoots to herbivory is usually not equal (Kaplan et al. 2008b).

Induced defense

Induced defenses are widely recognized as an adaption involving plant phenotypic plasticity to an unpredictable environment (Agrawal 2001; Kessler and Baldwin 2004). Induced defenses are primarily defined as plant responses to herbivore attack or pathogen infestation that can reduce damage and benefit the plants. These induced defenses can be highly valuable in maximizing plant fitness and balancing multiple (defense and growth) functions in the course of plant development (Boege and Marquis 2005). These functions can change in priority as environmental threats and the growth phase of a plant change.

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induction of secondary chemicals in foliage following root herbivory, leading to reduced leaf consumption by and growth of foliar herbivores. For example, Bezemer et al. (2003) found that root herbivory by wireworms on cotton plant roots reduced the relative growth rate of foliar caterpillars and their leaf consumption by inducing enhanced levels of alkaloids. The defense induced (termed "induced defense", box 1.3) by root herbivore on aboveground herbivores can however be specific both with respect to the inducer and with respect to the suite of herbivores that it affects (Box 1.4).

Box 1. 4 *Specificity of induction vs. specificity of effect*

Specificity of induction

Specificity of induction often occurs when herbivore species (damage types) differ in the type or strength of induction of defense in a given host plant (Hartley and Lawton 1987). For example, Agrawal (2000a) examined the specificity of induction using four lepidopteran herbivores feeding on wild radish plants and he found herbivores either caused strong induced defense, no induced defense or induced susceptibility. Specificity of induction can be caused by plant phenotypic (Traw and Dawson 2002) or chemical responses (van Zandt and Agrawal 2004; Chung and Felton 2011) to different herbivores.

Specificity of effect

Specificity of effect refers to differences between herbivore species in their response to a given defense induction (Stout et al. 1998). In the aforementioned study by Agrawal (2000a) they also explored the responses of herbivores to the induced defenses. Plant responses induced by some herbivores showed defense against all the herbivores while responses by other herbivores only defended against specific herbivores or showed no defense effects. Other studies comparing the colonization of herbivore-treated and untreated plants by later herbivores also frequently observed specificity of effects (Inbar et al. 1998; Agrawal and Sheffiffs 2001; Riihimaki et al. 2003; Viswanathan et al. 2005).

Root-feeding nematodes Nematodes are the most abundant root feeders of many plant species worldwide. They can substantially change plant size and quality (Stanton 1988), and thus influence plant palatability to concurrent herbivores in the other compartment (Kaplan et al. 2011). Given that root-feeding nematodes can reduce phloem amino acid contents it has been postulated that phloem-feeding insects like aphids and spider mites can be negatively influenced. This was confirmed in a study that showed that root-feeding nematodes reduced fecundity of aphid *Rhopalosiphum padi* by decreasing the content of amino acids in the plant phloem (Bezemer et al. 2005). Similarly, in a microcosm the sedentary endoparasitic nematode *H. schachtii* also reduced

the growth and fecundity of the aphids *Brevicoryne brassicae* and *M. persicae* feeding on *Beta vulgaris* and *Brassica oleracea* plants (Hol et al. 2013). However, a negative effect of root-feeding nematodes on aphids is not always shown. In a study using the root-feeding nematode *H. schachtii* inoculated to *A. thaliana* plants no effects on population growth of *B. brassicae* aphids were observed (Kutyniok and Müller 2012). Moreover, a recent study with thrips and spider mites feeding on the leaves of *A. thaliana* plants infected by the nematode *H. schachtii* showed that thrips tended to avoid nematode-infected plants while spider mites even preferred these plants (Kammerhofer et al. 2015).

In nematode-caterpillar systems, the outcome of the above-belowground interactions also seems to be highly variable (Wondafrash et al. 2013). Van Dam et al. (2005) reported that plant quality of black mustard was decreased by the root-feeding nematode *Pratylenchus penetrans*, which reduced the performance of *Pieris rapae* caterpillars feeding from the leaves of black mustard. In that study it was argued that these observed impacts were caused by an induction of glucosinolates and phenolics in plant foliage as a result of root herbivory. In contrast, a later study showed that root herbivory by the root-knot nematode *M. incognita* had a positive effect on foliar feeding caterpillars (*Spodoptera exigua*) of tobacco plants because nematode herbivory can negatively affect the production of nicotine in plant roots that can be transported up to shoots as a defense (Kaplan et al. 2008b). In other studies, infection of *M. incognita* had no significant impact on foliar levels of the defense compound gossypol in cotton (Olson et al. 2008), or the development of *Pseudoplusia includes* caterpillars on *Glycine max* plants (Carter-Wientjes et al. 2004).

Altogether the aforementioned studies show that root feeding nematodes can differ in impacts on foliar insect herbivores probably varying with nematodes species, susceptibility of the plant to herbivory, as well as characteristics such as feeding mode or specialism of the foliar feeding insects (Wondafrash et al. 2013).

Shoot herbivory impacts on root herbivore growth

For experimental convenience current studies on plant-mediated herbivore interactions mainly focused on propagation from root to shoot while studies on effects from shoot herbivory on root herbivores are rare. These studies neglected the fact that plants are equally colonized by belowground herbivores (Van Dam 2009). Thus far, there are relatively few studies providing evidence for effects of shoot herbivores on root feeders (Erb et al. 2008) but the limited number of available studies suggests a more predictable outcome than effect from the other propagation. Shoot damage by herbivory can increase the level of root defense compounds and tend to negatively influence root herbivores (van Dam and Heil 2011; Erb et al. 2015). Currently, the majority of studies on this topic focused on effects of aboveground insects and simulated damage (e.g. clipping) on root-feeding insects or root-feeding nematodes.

Shoot insects Tindall and Stout (2001) used fall armyworm to defoliate rice leaves and observed a strong reduction in the density and larval weight of root-feeding rice water weevils. Evidence is accumulating that shoot insect herbivory can induce systemic resistance in plant roots that cause negative effects on subsequent root herbivores (Bezemer and van Dam 2005). For example, feeding by the leaf chewer *Pieris brassicae* can result in higher levels of glucosinolates in the roots and thereby reduce the survival of the root insect larvae of *Delia radicum* (Soler et al. 2007). Similarly, leaf feeding by *Spodoptera frugiperda* can reduce the survival rate of later arriving western corn rootworms *Diabrotica virgifera* and this has been shown both in laboratory and field studies (Gill et al. 2011; Erb et al. 2011). However, the effects of shoot herbivory on root herbivores may not necessarily be negative. For instance, Erwin et al. (2014) reported that initial foliar herbivory facilitated the damage and growth of root larvae on milkweed plants. As adults and larvae of the red milkweed beetle *Tetraopes tetraophthalmus* were respectively used as above- and as belowground herbivores in this study, the authors suggested that facilitative effects may be more common between conspecific aboveground adult insects and their root-feeding larvae than between heterospecific insects.

However, a recent study (Milano et al. 2015) compared the effects of conspecific and heterospecific shoot herbivory on root herbivore performance and showed negative effects for both types of herbivores. In addition to systemically induced defense compounds as a result of foliar herbivory that can affect performance of root herbivores, there may also be alternative mechanisms involved in mediating effects between shoot and root herbivores. For example, Kaplan et al. (2008a) suggested that shoot herbivory can induce increased root sink strength that may also result in facilitation on root herbivores, such as root-feeding nematodes.

Grazing/clipping The effects of clipping or mammal grazing by ungulates on the growth or population size of root herbivores, e.g. root-feeding nematodes, have also been examined in several studies (Mikola et al. 2001; Bazot et al. 2005; Veen et al. 2010). These studies suggest that defoliation can induce plant reallocation of stored or newly assimilated resource from attacked tissues into storage organs like roots, termed "herbivore-induced resource sequestration" (Orians et al. 2011). These induced resource reallocations can stimulate plant root growth (Schon et al. 2010) and cause an increase in root-feeding nematode abundance. In addition, defoliation by herbivores can induce an increase of nitrogen in roots that provide higher food quality and also contribute to enhanced nematode abundance (Frost and Hunter 2008). In contrast, defoliation may also lead to a decline of root-feeding nematodes due to a decline in the plant's favorable microclimate (Ingham and Detling 1984) or may not alter root insect larvae, as in the case of *Phyllophaga sp.* survival and final biomass (Morón-Ríos et al. 1997).

Although the above studies suggest that shoot-feeding insects tend to show an overall negative effect on root-feeding herbivores, defoliation and mechanical clipping are relatively variable in their impacts on root herbivores. Therefore more empirical studies on impacts of defoliation and foliar clipping upon root herbivores growth are needed for a general conclusion.

Box 1.5 Priming effects

Priming

The state of priming is defined as a physiological condition in which plants are able to more strongly or rapidly mount defense responses to future (a)biotic stresses (Conrath et al. 2006, 2015). Priming allows plants to reduce costs of implementing defense in the absence of attack. A primed state in the plant can be provoked both by natural and synthetic compounds, such as jasmonic acid (JA), salicylic acid (SA), and β-aminobutyric acid (Worrall et al. 2012). In the context of plant-herbivore interactions, plants can be primed for both direct and indirect defenses. For example, a study by Ton et al. (2007) showed that maize plants that had been previously exposed to *Spodoptera littoralis* showed a stronger up-regulation of defense gene expression and a stronger reduction of the relative growth rate of conspecific caterpillars upon subsequent attack by this herbivore than plants that had not been exposed to previous herbivory. Further, they showed that plants primed by *S. littoralis* released a higher abundance of Herbivore-Induced Plant Volatiles and more strongly attracted the parasitoids of these caterpillars.

Mechanisms of priming

Priming can be based amongst others on elevation of pattern recognition receptors, dormant signaling enzymes, transcription factor activity, and alterations in chromatin state (Conrath et al. 2015).

AMF-plant-aboveground herbivore interactions

Plant-mediated interactions between above- and belowground communities also include players other than herbivores. Some organisms like arbuscular mycorrhizal fungi (AMF) that have different ecological niches can also interact with plants and herbivores (Gehring and Whitham 2002). Mycorrhizal fungi can colonize more than 80% of all terrestrial plant species and provide crucial functions in soil nutrient uptake and plant defense (Smith and Read 2010).

AMF can interact with aboveground herbivores via multiple mechanisms (Bennett et al. 2006; Gehring and Bennett 2009). They can either positively influence aboveground herbivores through improving plant vigor and foliar nutrient concentrations (Borowicz 1997) but also negatively affect foliar herbivores through changes in constitutive and inducible defenses against herbivory (Bennett et al. 2006). Indeed mycorrhizal colonization can significantly increase the concentration of defense compounds, including iridoid glycosides (Gange and West 1994) and alkaloids (AbuZeyad et al. 1999) that can strongly reduce performance and growth of some aboveground herbivores. Other studies have suggested that plant root colonization by AMF can also enhance the production of volatile organic compounds attractive to aphids, potentially benefiting these herbivores (Babikova et al. 2014). In addition, association with AMF can indirectly influence foliar herbivores by mediating plant attraction of enemies of herbivores (Gehring and Bennett 2009). However, this is not always the case as both decreases and increases of enemy attraction on mycorrhizal plants have been reported (Gange et al. 2003; Laird and Addicott 2007; Schausberger et al. 2012).

Although studies on mycorrhizae-plant-insect interactions have been accumulating, a substantial gap still remains in understanding mycorrhizal functions in regulating herbivorous insect populations (Reidinger et al. 2012). Most studies have reported a negative effect of colonization by mycorrhizae on the performance of generalist chewing insects, but a positive effect on the performance of generalist sucking insects and specialists (Koricheva et al. 2009). The reduced performance of generalist chewing insects is deemed to be the consequence of the priming of plants by mycorrhizae for jasmonic acid (JA)-related defense compounds (Box 1.5, Pozo and Azcón-Aguilar 2007), whereas the facilitation of sap-sucking insects is thought to result from mycorrhizal suppression of salicylic-acid (SA)-related defenses due to negative crosstalk between the JA and SA signaling pathways (Pozo and Azcón-Aguilar 2007; Jung et al. 2012). Given that most of these studies have mainly focused on the influence of mycorrhizae on the colonization, survival, growth or reproduction of individual aboveground herbivores, studies

examining the composition and dynamics of aboveground insect communities may be a priority (Reidinger et al. 2012).

Temporal patterns in aboveground-belowground herbivore interactions

Time course of induced defense

Plants in natural communities can be subjected to repeated damage by herbivores during their lifetimes (Underwood 2012). Most plants can respond to such damage with chemical or physiological changes that influence defence against current or future attacks (Underwood 1998; Gomez et al. 2010). The time lag between damage and the onset of defense, as well as the time lag between the cessation of damage and the relaxation of defense are supposed to be crucial in determining the effectiveness and costs of induction (Karban and Baldwin 1997; Gomez et al. 2010; Karban 2011). Therefore, it is important to know how fast a plant can induce resistance to reach an effective level of defense, and it is important to establish how long the defense can be maintained.

Although it has been long established that the effectiveness of induced responses in plant-insect interactions is highly related to their temporal pattern (Green and Ryan 1972; Haukioja 1980; Nykänen and Koricheva 2004; Karban 2011), few studies have fully characterized the time course of these responses (Karban 2011). Several studies have shown that the time course may not simply follow a single trajectory. For example, Underwood (1998) found that soybean under attack by Mexican beetles showed a higher resistance to subsequent feeding than undamaged plants. But this induced defense only lasted up to 15 days after the initial damage, and after 20 days damaged plants tended to be more susceptible than undamaged plants. Similar findings were reported for tobacco (Baldwin 1989), cotton (McAuslane et al. 1997), and cucumber (Agrawal et al. 1999). Accordingly, inducible defense dynamics tend to show a bell-shaped curve characterized by an initial build-up followed by a short peak and then a gradual decrease (Schultz 1988; Underwood 1998; Gomez et al. 2010). This pattern was further observed by Agrell et al. (2003)

who found that feeding larvae preferred undamaged plants to those plants damaged 5 or 7 days earlier, but showed no preference 1, 9, and 14 days after damage. More importantly, even for the induction of responses of trees, that usually takes much longer time, this basic pattern can still be identified. For instance, *Acacia drepanolobium* produced larger spines under browsing two months after damage, but relaxed this induced trait after five years (Young and Okello 1998; Young et al. 2003).

The above-mentioned studies show that the time course of induction follows a clear general pattern. However, the mechanisms behind this pattern are not clear at all. It was generally supposed that the time interval between herbivore damage and the first symptoms of induced defense is used for synthesizing and producing detectable amounts of induced chemicals (Agrell et al. 2003; Karban 2011). This may be true for chemical induction, but not necessarily for induction of structural defenses, which are more dependent on the period needed for growing new tissues (Young et al. 2003). In addition, Björkman et al. (2008) reported that willows produced new leaves with higher density of trichomes 10-20 days after damage by adult leaf beetles *Phratora vulgatissima*, which coincided with the onset of feeding by beetle offspring. This shows that cues indicating whether the risk of damage continues or not is also involved in determining the time course of induction and the time lag of relaxation (Young et al. 2003). In contrast, the time lag between cessation of damage and relaxation of induced defense may be associated with costs and benefits of maintaining defensive traits (Gomez et al. 2007, 2010). For example, Gomez et al. (2007) showed that maintaining systemically induced defense over a period of 3 weeks has rather low costs.

Sequence of AG and BG herbivory

While it is evident that the location of feeding may affect the interactions between insect herbivores, the sequence of colonization on plants by these herbivores was also shown to play an important role (Van Dam and Heil 2011; Soler et al. 2012). A recent meta-analysis showed that the sequence of arrival of aboveground and belowground herbivores could significantly influence

the outcome of their interactions (Johnson et al. 2012). Interestingly, these sequential effects depend on whether the interaction propagated from aboveground to belowground parts or *vice versa*. In the meta-analysis of Johnson et al. (2012), belowground herbivores were significantly reduced only when aboveground herbivores were introduced first, and not when they were introduced simultaneously or later than the belowground herbivore. When the effects of belowground herbivores were studied on aboveground herbivores, simultaneous introduction of both aboveground and belowground herbivores resulted in a significant increase in the performance of aboveground herbivores. When the belowground herbivores were introduced before or after the aboveground herbivores, root herbivores did not affect aboveground herbivores. Notably, the meta-analysis of Johnson et al. (2012) tested the significance of several sequential effects, but these effects were examined separately in different studies by using different plant-insect systems. Therefore, it is still necessary to fundamentally identify the importance of the propagation direction and sequence of occurrence in aboveground and belowground interactions, as well as the mechanisms involved.

Recently, Erb et al. (2011) examined in a single study whether sequential arrival (before, simultaneous or after) of the leaf chewer *Spodoptera frugiperda* on maize plants affected the performance of the root-feeder *Diabrotica virgifera*. The authors reported that *S. frugiperda* feeding on aerial parts of maize greatly reduced the colonization of root-feeding *D. virgifera* in the field and their weight gain in the laboratory, but only when *S. frugiperda* arrived on the plants before the root feeder. The shoot herbivore did not significantly affect the performance of root herbivores that were already feeding when the aboveground herbivore arrived. Hence, the authors hypothesized that the leaf chewer might have increased the level of some feeding-deterrent compounds that interfered with host location of new colonizers but did not necessarily influence the feeding behavior of root herbivores that had already colonized the plant. In addition, Johnson et al. (2012) hypothesized that the sequential effect of the leaf chewer on the weight gain of the root feeding larvae that was observed in the study of Erb et al. (2011) could be due to induction or priming

of defense compounds that reduced growth of the root feeders. Priming is a physiological process initiated within plants in response to an environmental stress (biotic or abiotic) that enables plants to respond more quickly and efficiently to future challenge (Frost et al. 2008). In contrast to induction, priming does not immediately result in upregulation of defense metabolites, but brings plants in a state "getting ready for battle" so that they respond with a faster and stronger defensive response upon subsequent attack (Conrath et al. 2006; Frost et al. 2008). Priming thus is an economical way to defend against subsequent attacks when a plant has previously been exposed to an attack.

Time course of induction in AG-BG interactions

Herbivory-induced responses can systemically cross the border of the soil surface and strongly regulate interactions between aboveground and belowground herbivores (van Dam et al. 2003; Bezemer and van Dam 2005; Kaplan et al. 2008a; van Dam and Heil 2011). For example, the shoot part of *Brassica nigra* whose roots were subjected to larvae of cabbage root fly (*Delia radicum*) had higher levels of glucosinolates and experienced less attack by aboveground herbivores (Soler et al. 2009). Similarly, herbivory on the aerial parts of *Brassica nigra* by *Pieris brassicae* significantly increased the level of the allelochemicals in roots and further reduced the survival of the root feeder *Delia radicum* and development of parasitoids of the root feeder (Soler et al. 2007). Given the significance of induced plant defense in the field of above-belowground research (van Dam and Heil 2011; Soler et al. 2012), integrating the timing of plant defense in an above-belowground context may be of importance. Thus far, however, few studies have attempted to record the dynamics of induced defense in one compartment of a plant initiated by herbivory on the other compartment. A study by van Dam and Raaijmakers (2006) showed that the root herbivore *Delia radicum* systemically induced shoot glucosinolates in *Brassica nigra* and *Brassica oleracea*, and the induced chemicals steadily increased until 14 days after damage. Similarly, the undamaged ramets of the stoloniferous herb *Trifolium repens* systemically became defense-induced 38-51 h after the initial local attack by *Mamestra*

brassicae larvae, and maintained this state of defense for at least 28 days (Gomez et al. 2010).

Although these studies were not based on manipulation within an above-belowground context, they show the potential and implication of checking the time course of induced defense in above-belowground interaction research, especially in the case of repeated damage or future attack in a natural community. This is because plants are usually subjected to repeated damage by herbivores either above or belowground during their lifetime (Karban 2011; Underwood 2012). Thus, it is significant to record the time course of induced defense at an event of attack by aboveground or belowground herbivory in order to predict the magnitude and frequency of future attack. While induction of defenses between initial and subsequent damage have been extensively documented (Underwood 2012), the majority of these studies only measured the defense level at one particular time point during the whole time course of the period (Karban 2011). In a recent study, Underwood (2012) repeatedly employed *Spodoptera exigua* on tomato plants to examine whether the repeated damage could restructure the time profiles of induced defense. The author found that herbivory needed 1 day for eliciting the defense but 15-20 days to relax it, which indicated that the defenses may be still active during a second damage event. This is also very important for the above-belowground interaction research when both foliar and root herbivores are sequentially or simultaneously employed to show how they influence each other during the two damage events (Poelman et al. 2008; Erb et al. 2011).

A comprehensive characterization of the time course of induced defense may not only deepen our understanding of the temporal features of aboveground and belowground interactions at a short term, but also contribute to our understanding of how cost and benefits of induced responses in plants are balanced and can shape selection on this trait.

Timing of AMF-herbivore interactions

Timing of mycorrhizal colonization may be crucial for the outcome of AMF-herbivore interactions because early mycorrhizal presence can prime plants for resistance to feeding by later arriving herbivores (Jung et al. 2012) by inducing a state "ready for battle". As priming and the primed state can be costly (Frost et al. 2008) the primed state of plants may relax over time in the absence of attacks from an economical perspective to maximize plant fitness (Stamp 2003). Thus, a temporal pattern of priming may be of interest to explicitly predict plant-insect interactions. Worrall et al. (2012) exposed tomato seeds with the priming agents jasmonic acid and β-aminobutryric acid to generate priming effects. They observed enhanced defense gene expression in primed plants upon later attack even 8 weeks after the treatments. Similar results were also reported by Rasmann et al. (2012) who used caterpillars to challenge *Arabidopsis* plants and observed induced resistance based on priming of jasmonic acid-dependent defense responses. These responses caused later caterpillar to grow 50% smaller and the primed state could even last until the next generation of plants. Moreover, a recent review of defense priming shows that a primed defense state can be inherited epi-genetically from defense-expressing plants (Pastor et al. 2013). AMF can prime plants and the primed responses can be systemically expressed in shoot tissues (Pozo et al. 2010). Similar to priming induced by insects and pathogens, AMF-induced priming is JA-dependent, since JA-responsive genes were induced earlier and to a higher extent in plants colonized by *Glomus mosseae* upon subsequent attack (Pozo et al. 2009; Song et al. 2013). We are not aware of any studies examining the time course of AMF-induced priming of herbivore defense and its consequences on plant herbivores.
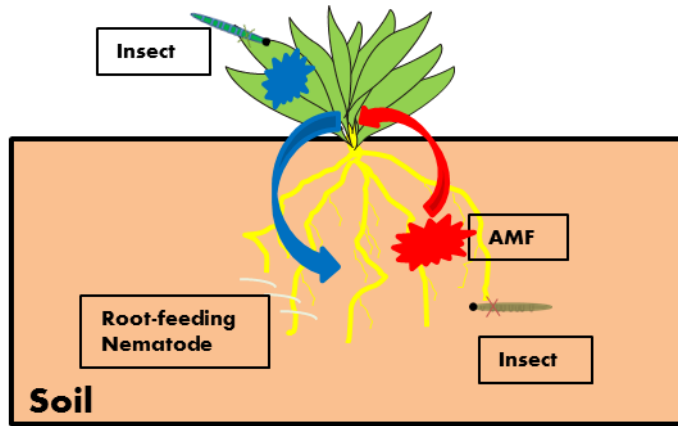
Figure 1.1 Schematic diagram of simplified above-belowground interactions studied in this thesis. Aboveground herbivory can systemically induce defense in roots and deter the feeding by belowground insects and *vice versa* (chapter 2). AMF colonize plant roots and can induce defense or prime plants to confer a stronger/quicker induced defense in foliage and reduce performance of particular insect groups (chapter 3). Aboveground herbivores can also induce plant resource allocation and defenses within individual plants (chapter 4) and may control root-feeding nematode reproduction and population size (chapter 5). All these interactions are examined from a temporal perspective that will be presented in detail in later chapters.

Aims of this thesis

This thesis aims to examine temporal aspects of interactions between aboveground herbivores and belowground organisms. I used a series of controlled experiments to understand how aboveground herbivores indirectly interact with belowground herbivores when they colonize the plants in different sequences and at different time points (Figure 1.1). In particular I investigate (1) how sequences of arrival on plants by aboveground and belowground herbivores affect their interactions, (2) how mycorrhizal fungi mediate the dynamics of plant defense induced by conspecific herbivory, (3) how simulated defoliation and nematode herbivory at different plant ages alter plant defense and growth responses and (4) how timing of simulated defoliation influenced the dynamics of root-feeding nematodes.

Outline of the thesis

In chapter 1, I introduced the general background information about above-belowground interactions with emphasis on temporal approaches used in above-belowground herbivore relations.

Chapter 2 focuses on the effects of sequential introduction of above- and belowground herbivores on each other to see whether the results of their interactions depend on when they arrive on the host plants. I measured the weight gain of herbivores, leaf consumption, and levels of plant defense chemicals in order to reveal the sequential effects observed in this chapter.

In chapter 2, I only explored above-belowground herbivore interactions at a given time of 4 days between the initiation and onset of herbivory. In chapter 3, I examine the dynamics of defense magnitude following foliar herbivory during an experimental time series. Given that AMF are known to prime plants for enhanced defense against chewing herbivores, I test whether the timing of defense responses to herbivory is altered by the presence of AMF.

In chapter 4, I used clipping to simulate aboveground defoliation and used root-feeding nematodes as belowground herbivores. I clipped plant foliar tissues when plants are at different ontogenetic stages to examine whether and how plants differently respond in terms of nutritional and chemical quality during ontogeny.

Following chapter 4, I test whether plant root-feeding nematode populations can accordingly respond to plant physiological changes induced by simulated defoliation at different ontogenetic stages in chapter 5.

In chapter 6 I discuss all the findings in this thesis and suggest further research priorities of above-belowground interactions using a temporal approach.

Chapter 2

Sequential effects of root and foliar herbivory on aboveground and belowground induced plant defense responses and insect performance

Minggang Wang, Arjen Biere, Wim H. Van der Putten, T. Martijn Bezemer

Abstract

Plants are often simultaneously or sequentially attacked by multiple herbivores and changes in host plants induced by one herbivore can influence the performance of other herbivores. We examined how sequential feeding on the plant *Plantago lanceolata* by the aboveground herbivore *Spodoptera exigua* and the belowground herbivore *Agriotes lineatus* influences plant defense and the performance of both insects. Belowground herbivory caused a reduction in the food consumption by the aboveground herbivore independent of whether it was initiated before, at the same time, or after the aboveground herbivore. By contrast, aboveground herbivory did not significantly affect belowground herbivore performance, but significantly reduced the performance of later arriving aboveground conspecifics. Interestingly, belowground herbivores negated negative effects of aboveground herbivores on consumption efficiency of their later arriving conspecifics, but only if the belowground herbivores were introduced simultaneously with the early arriving aboveground herbivores. Aboveground-belowground interactions could only partly be explained by induced changes in an important class of defense compounds, iridoid glycosides (IGs). Belowground herbivory caused a reduction in IGs in roots without affecting shoot levels, while aboveground herbivory increased IG levels in roots in the short term (four days) but only in the shoots in the longer term (seventeen days). We conclude that the sequence of aboveground and belowground herbivory is important in interactions between aboveground and belowground herbivores and that knowledge on the timing of exposure is essential to predict outcomes of above-belowground interactions.

Introduction

Virtually all plants in nature are exposed to herbivory by a variety of aboveground and belowground insect species. Insect herbivory can elicit morphological, physiological, and biochemical plant responses, which can depend greatly on the identity of the attacker (Karban and Baldwin 1997). Such insect-induced changes in plant defenses can subsequently influence the performance of the insect that causes the feeding damage on the plant. Moreover, via feeding-induced changes in the plant, an insect herbivore can also influence the performance of other insects that feed on the same plant (Kaplan and Denno 2007). These plant-mediated effects between insect herbivores can be positive and negative for one or both of the herbivores (Kaplan and Denno 2007).

Most studies that have examined inter- or intraspecific interactions between insect herbivores on a shared host plant have focused on aboveground insects. However, plants are also frequently attacked by belowground insects, and many studies have shown that aboveground and belowground insects can influence each other via changes in the shared host plant (Masters et al. 1993; Van der Putten et al. 2001; Bezemer et al. 2003; Wardle et al. 2004; Van Dam et al. 2006; Kaplan et al. 2008a). These effects can be mediated by changes in concentrations of primary compounds in the plant such as nitrogen or carbon (Masters et al. 1993). Moreover, root and shoot feeding insects on a plant can influence each other's performance via systemic induction of secondary (defense) compounds in the plant (Bezemer et al. 2003, 2004; Bezemer and Van Dam 2005; Kaplan et al. 2008a).

Interactions between insect herbivores can occur when they feed simultaneously on a plant, but insects can also affect each other when they feed sequentially on the same plant (Maron 1998; Blossey and Hunt-Joshi 2003; Voelckel and Baldwin 2004). Interestingly, several studies have shown that concentrations of defense compounds in a plant may only change in response to the first attacker and may not be modified by later arrivers (Viswanathan et al. 2007; Poelman et al. 2008; Erb et al. 2011; Gomez et al. 2012). As the

defense response of a plant strongly depends on the identity of the first attacker (Voelckel and Baldwin 2004; Viswanathan et al. 2007), the performance of an insect on a plant may depend both on the identity of the other herbivores on that plant, and on when they have been feeding on the plant (Poelman et al. 2008; Gomez et al. 2012).

Recently, Johnson et al. (2012) concluded in a meta-analysis of 35 studies that the interaction between aboveground and belowground insect herbivores also depends on the sequence of aboveground and belowground herbivory. Aboveground herbivory negatively influenced the performance of belowground insect herbivores, but only when aboveground herbivores were feeding first. In contrast, belowground herbivores appeared to facilitate the performance of aboveground herbivores, but only when they fed simultaneously; introduction before or after the aboveground herbivore did not result in facilitative effects of belowground herbivores (Johnson et al. 2012). In the meta-analysis sequential effects of aboveground and belowground herbivore additions were compiled from many different aboveground and belowground insect species that were feeding on a variety of different plant species. This leaves the question unanswered how sequential feeding within single host-aboveground-belowground herbivore systems may influence all individual components. However, such studies are rare. One exception is a study by Erb et al. (2011) who reported that the leaf chewer *Spodoptera frugiperda* negatively affected the performance of the root chewer *Diabrotica virgifera* on maize, but only when the aboveground herbivore started feeding on the plant before the belowground herbivore.

Several studies have suggested that in response to simultaneous aboveground and belowground herbivory, plant defense compounds may increase more in shoot than in root tissues (e.g. Bezemer et al. 2004; Rasmann et al. 2009, Erb et al. 2009). Similarly, simultaneous application of jasmonic acid to roots and shoots of Brassicaceous plants as a mimic of herbivory by chewing insect herbivores increased glucosinolate concentrations in shoots but not in roots (Van Dam et al. 2004). Via these effects on plant defenses, simultaneous aboveground and belowground herbivory may alter the performance of other

herbivores in ways that may not be predictable from their effects when they differ in their time of appearance (Bezemer et al. 2003; Erb et al. 2009; Rasmann et al. 2009). We are not aware of any study that has examined how sequential feeding by both aboveground and belowground herbivores affects plant defenses and the performance of later-feeding herbivores.

In the present study, we examined how sequential feeding on ribwort plantain (*Plantago lanceolata* L., Plantaginaceae) by aboveground and belowground insect herbivores may influence aboveground and belowground plant biomass and defense responses, as well as insect herbivore performance. *Plantago lanceolata* is a short-lived perennial forb that produces a wide range of allelochemicals (Sutter and Müller 2011). Two important defense chemicals present in *P. lanceolata* are the iridoid glycosides (IGs) aucubin and catalpol. Numerous studies have shown that the concentrations of these compounds in shoots and roots can have strongly negative effects on the performance of generalist above- and belowground insect herbivores, but that they can be used as feeding cues and oviposition stimulants by specialist insect herbivores (e.g. Bowers and Puttick 1989; Nieminen et al. 2003; Wurst et al. 2008; Reudler Talsma et al. 2008, 2011). Moreover, these compounds affect the performance of both plant-beneficial and plant-pathogenic fungi (Marak et al. 2002b; Biere et al. 2004; De Deyn et al. 2009). Several studies have shown that iridoid glycoside concentrations can increase in response to damage by aboveground (e.g. Bowers and Stamp 1993; Darrow and Bowers 1999; Marak et al. 2002a) and belowground (e.g. Wurst et al. 2008) insect herbivores. Herbivory can cause both a local increase in IG concentrations in the damaged plant tissues, and a systemic induction in aboveground and belowground plant parts (Darrow and Bowers 1999; Marak et al. 2002a; Wurst et al. 2008), providing the scope for mediation of interactions between aboveground-and belowground organisms associated with the plant.

We tested the hypotheses that (i) aboveground herbivory will cause a systemic increase in IG concentrations in aboveground and belowground plant tissue and will reduce the performance of aboveground and

belowground insect herbivores, but only when the aboveground herbivore is introduced first; (ii) belowground herbivory will positively affect aboveground insect herbivore performance, but only when they start feeding simultaneously; (iii) when both an aboveground and a belowground herbivores are introduced prior to a later arriving aboveground herbivore, their effects on the later arriving herbivore depend on their sequence of introduction.

Materials and methods

Plants and insects

Plantago lanceolata L. (Plantaginaceae) (ribwort, plantain) is a plant species with a world-wide distribution that serves as a model plant species in plant-insect interaction research (e.g. Bowers and Stamp 1992). *Plantago lanceolata* contains iridoid glycosides, predominantly aucubin and catalpol, which are toxic or deterrent to generalist herbivores but act as feeding and oviposition cues for specialists. Seeds of *Plantago lanceolata* were purchased from a wild seed supplier (Cruydt-hoeck, Nijeberkoop, The Netherlands). The seeds were surface sterilized with sodium hypochlorite (1%), sown on glass beads and placed in an incubator (16h light, 20/25°C night/day temperature). Germinated seedlings were transplanted into 1.1 liter pots (1 plant per pot) filled with 1100 g sandy-loam mineral soil (particle size distribution with 3% < 2 μm, 17% 2-63 μm, and 80% > 63 μm; RH = 12.3%) collected from a restoration grassland (De Mossel, Ede, The Netherlands) where *P. lanceolata* abundantly occurs. In the laboratory the soil was sieved through a 1 cm mesh, homogenized and gamma-sterilized (>25KGray). Pots were placed randomly in a greenhouse. Plants were watered 3 times per week and randomly redistributed within the greenhouse once a week. Natural daylight in the greenhouse was supplemented by 400 W metal halide lamps (225 μmol m^{-2} s^{-1} PAR).

Wireworms are larvae of the click beetle *Agriotes lineatus* L. (Coleoptera: Elateridae). They are generalist root herbivores commonly found in

grasslands and a pest of many cultivated crops (Parker and Howard 2001). Wireworms typically stay in the soil for 3 to 6 years as larvae before pupation (Parker and Howard 2001). Wireworms were purchased from Applied Plant Research (PPO-Wageningen University and Research Centre), Lelystad, The Netherlands. Before introduction, they were starved for 3 days in moist soil and weighed using a microbalance.

Spodoptera exigua H. (Lepidoptera: Noctuidae), the beet armyworm, is a generalist foliar herbivore that feeds on plants from more than 30 families (Merkx-Jacques et al. 2008). It originates from Southeast Asia, but nowadays has a world-wide distribution. The larvae go through five instars during development (Harvey et al. 2005). Beet armyworm eggs were obtained from the Laboratory of Entomology, Wageningen University, The Netherlands and reared until the third instar on an artificial diet (Singh 1983). Rearing took place in a growth chamber (24°C, 16:8-hr light/dark photoperiod, 70% RH) before being introduced on the plants.

Experiment 1: Impact of the sequence of aboveground herbivore introduction on belowground herbivore performance

To determine the effects of the sequence of aboveground herbivore introduction on plant growth and defense and on the performance of a belowground herbivore, an experiment was set-up with 66 pots. One seedling was planted into each pot. Twenty-eight days after planting (t = -5), all plants were caged using cylindrical mesh cages (height 1 m, diameter 35 cm). Six treatments were initiated (Figure 2.1a), with 11 replicate pots per treatment. The treatments were: (1) the aboveground herbivore was introduced at t = -5, five days before introduction of the belowground herbivores (A>B); (2) simultaneous introduction of aboveground and belowground herbivores at t = 0 (A=B); (3) the aboveground herbivore was introduced at t = 5, five days after introduction of the belowground herbivores at t = 0 (B>A); (4) introduction of belowground herbivores at t=0 without aboveground herbivores (B); (5) introduction of the aboveground herbivore at t = 0 without

belowground herbivores (A); and (6) control plants without aboveground and belowground herbivores (Co).

As belowground herbivores, two pre-weighed late-instar (mean = 24.6 mg; SE = 0.7 mg) *A. lineatus* were placed into 1-cm-deep small holes made in the soil 33 days after transplantation (t = 0). All wireworms immediately burrowed into the soil. Similar holes were also made in the soil of pots without wireworms. The aboveground herbivore treatment consisted of one third-instar *S. exigua* per pot (mean = 49.2 mg; SE = 0.1 mg). The larva could move freely on the plant within the cage.

Plants were harvested at t = 17 (17 days after introducing the belowground herbivores in treatments 1-5). At harvest the aboveground insects were removed from the plants, and the aboveground biomass was clipped at ground level. Wireworms were recovered from the soil, reweighed and the weight gain of each wireworm was determined. The fifth youngest leaf and a subsample of roots of 11 randomly chosen plants assigned to each of the treatments A, B, B=A and Co were removed with a razor blade, immediately frozen at -20 °C, freeze-dried for 4 days under vacuum (-55 °C collector temperature, Labconco Free Zone 12 L Freeze Dry System, USA), weighed and ground. Twenty five mg of each sample was extracted overnight in 70% methanol, then filtered and diluted 10 times with ultrapure water. The concentration of the IGs aucubin and catalpol were analyzed using HPLC as described by Marak et al. (2002b). Due to practical limitations, leaf and root chemistry could only be determined for a subset of the treatments. The remaining aboveground and belowground biomass of each plant was oven-dried at 70°C and dry weight was determined.

The effect of aboveground herbivory on wireworm performance was first analyzed independent of sequence (treatments A>B, A=B, B>A vs. B) using one-way ANOVA. Subsequently, we carried out a one-way ANOVA in which we analyzed all four treatments, using a Dunnett *post hoc* test to compare treatment B with each of the treatments A>B, A=B, and B>A. The effect of the timing and duration of aboveground herbivory on wireworm performance

was also analyzed using linear regression with the duration of aboveground herbivore feeding on plants as continuous variable (0 days for treatment B, 12 days for B>A, 17 days for A=B, and 22 days for A>B). Total plant biomass and shoot-to-root ratio were analyzed using one-way ANOVA for the effects of aboveground (A vs. Co), belowground (B vs. Co) and combined above and belowground herbivory (A>B, A=B, B>A vs. Co). The latter was again followed by a Dunnett *post hoc* test in which the three combined herbivory treatments were each compared with control plants without herbivory. IG concentrations were analyzed using two-way ANOVA with presence or absence of aboveground herbivory and belowground herbivory as main factors. All data were checked for normality using a Kolmogorov-Smirnov one-sample test and for homogeneity of variance using a Levene test before analysis.

Experiment 2: Impact of sequence of belowground and aboveground herbivore introduction on aboveground herbivore performance

To examine the effects of the introduction sequence of aboveground and belowground herbivores on aboveground herbivore performance, we set up an experiment with 221 pots. We used fourth instar *S. exigua* larvae (1 per clip-cage) as aboveground and wireworms (1 per pot) as belowground "treatment" herbivores and *S. exigua* larvae as aboveground response herbivores. To standardize the amount of damage caused by the aboveground "treatment" herbivores, one *S. exigua* larvae was introduced into a 2.0-cm diameter clip-cage that was placed on the top area of a fully-expanded mature leaf (one clip-cage per plant). After one day, when the entire area within the clip-cage was eaten, the clip-cage was moved to another mature leaf so that on each plant there were two areas of 3.14 cm^2 consumed over a period of two days. At t = 0 (Figure 2.1b; 31 days after transplanting), all plants were caged and in 204 cages (all treatments except the Co treatment, see below) one pre-weighted third instar (mean = 27.2; SE = 0.4 mg) *S. exigua* was introduced. These larvae were considered aboveground response herbivores (labeled as "S"). The response herbivores could move freely on the plant. Eight days after

introducing the aboveground response herbivores, they were collected from each cage, re-weighed and weight gain was calculated.

The experiment was set up with 13 treatments and 17 replicate pots per treatment. In all treatments except treatment 13, the response herbivore (S) was introduced at t=0. The treatments were (Figure 2.1b): (1) the AG treatment herbivore was introduced four days before (A>S), (2) at the same time (A=S), and (3) four days after the response herbivore (S>A). The BG treatment herbivores were introduced (4) four days before (B>S), (5) at the same time (B=S), and (6) four days after the response herbivore (S>B). The AG and BG treatment herbivores were both introduced (7) four days before (AB>S), (8) at same time (AB=S), and (9) four days after the response herbivore (S>AB). To determine how the relative sequence of prior aboveground and belowground herbivory influenced the performance of the response herbivore, the aboveground treatment herbivore was introduced (10) four days before the belowground treatment herbivore and the aboveground response herbivore (A>BS), (11) the belowground herbivore was introduced four days before the aboveground treatment herbivore and the response herbivore (B>AS). Finally, (12) the response herbivore was introduced without other herbivores (S), and there was a control (13) without aboveground and belowground herbivory (Co).

Forty extra plants were grown to determine the effects of aboveground and belowground herbivory on IG concentrations in the plant at the time that the response herbivore was introduced on the plant. Treatments included four days of aboveground (A), belowground (B), or aboveground and belowground herbivory (AB), and no herbivory (Co) with 10 replicate plants per treatment. All herbivory treatments for these extra plants were initiated at t = -4 and the plants were harvested at t = 0. The fifth leaf and a subsample of roots was freeze-dried and analyzed as described for experiment 1. All other plants were harvested at t = 8, eight days after the response herbivore was introduced. Roots were removed carefully from the soil and rinsed. All leaves of each plant were scanned using a photo scanner (EPSON, PERFECTION 4990, Japan) and the leaf area consumed by the response

herbivore was determined using the software WinFOLIA (Regent Instruments, Sainte-Foy, Canada). Consumption efficiency was calculated as weight gain of *S. exigua* per consumed square centimeter leaf area. As the *S. exigua* were reared on a moist artificial diet until introduction on the plant, the initial weight of the larvae was adjusted to compensate for the 30% moisture loss after introduction (Boldt et al. 1975). Shoot and root biomass was then oven-dried and total biomass was determined.
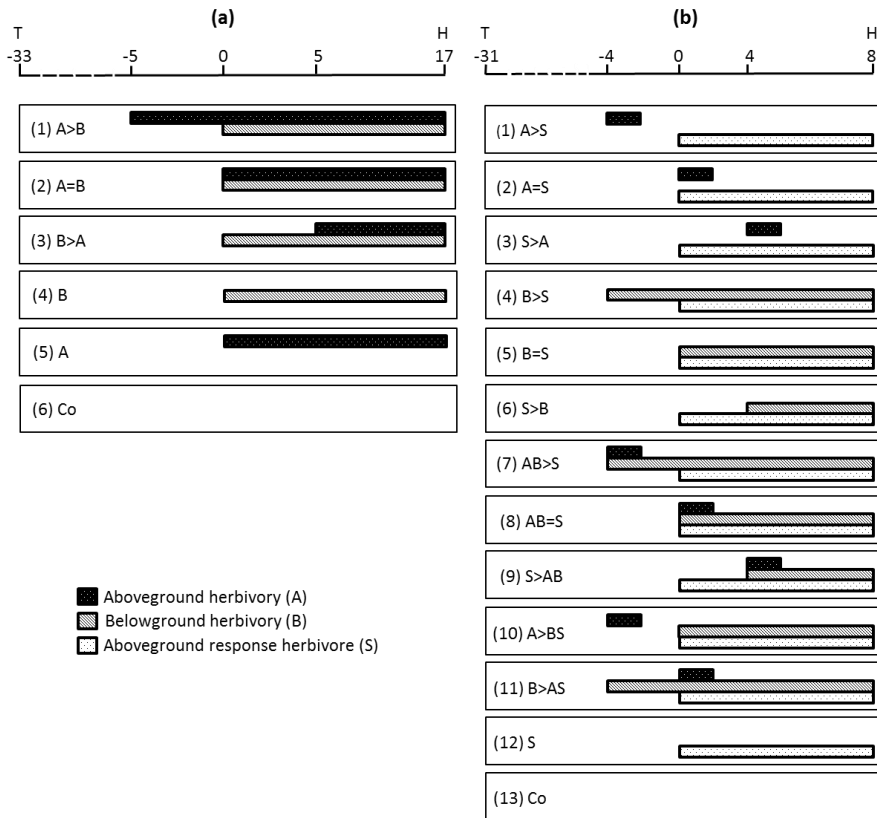
Figure 2.1 Experimental design of (**a**) experiment 1 and (**b**) experiment 2. Response herbivores *Agriotes lineatus* (experiment 1) and *Spodoptera exigua* (experiment 2) were introduced at t = 0. The horizontal bars indicate when the aboveground and belowground herbivores were feeding, and the length of each bar represents the feeding duration. T = - 33 and H = 17 (**a**) or T = -31 and H = 8 (**b**) indicate the day of transplantation and harvest, respectively.

To determine the influence of belowground herbivory on the weight gain, leaf area consumption, and food consumption efficiency of the response herbivore *S. exigua*, we performed a one-way ANOVA testing effects of belowground herbivory (treatments 4-6) against the treatment with the response herbivore only (treatment 12). As in experiment 1, a one-way ANOVA was followed by a Dunnett *post hoc* test in which each of the treatments B>S, B=S, S>B were contrasted with S, to examine whether the significance of the effect of belowground herbivores depended on their introduction sequence. Similar analyses were performed for the effects of aboveground herbivory (treatments 1-3 vs. 12) and simultaneous above- and belowground herbivory (treatments 7-9 vs. 12). Effects of the joint herbivory by response caterpillars and aboveground, belowground, or aboveground plus belowground herbivores on plant biomass and shoot-to-root ratio were also analyzed using one-way ANOVAs using undamaged plants (treatment 13) as a control.

To determine the effect of sequential introduction of aboveground and belowground treatment herbivores on the aboveground response herbivore, the treatments 7, 8, 10 and 11 were analyzed using two-way analysis of variance, with the sequence of aboveground herbivory (before and simultaneous) and belowground herbivory (before and simultaneous) as main factors. IG concentrations in root and shoot tissues were analyzed using two-way ANOVA with presence or absence of aboveground and belowground herbivory as main factors. All data were checked for normality and homogeneity of variance before analysis.

Results

Experiment 1: Impact of the sequence of aboveground herbivore introduction on belowground herbivore performance

There was no overall significant effect of the aboveground herbivore treatments on the mean weight gain of wireworms ($F_{1,39}$ = 2.16; P = 0.150; Figure 2.2a). Wireworms on average gained more weight when *S. exigua* had been introduced before the wireworms, and with increasing duration of

aboveground herbivore feeding, but these effects were not statistically significant (Dunnett test, $P = 0.086$, and $F_{1, 41} = 3.83$; $P = 0.057$; Figure 2.2a, respectively). Total plant biomass was significantly reduced by combined aboveground and belowground herbivory ($F_{1,40} = 8.40$; $P = 0.006$; Figure 2.2b), but this effect was only significant for the longest feeding duration of *S. exigua* (22 days; Dunnett test; $P = 0.002$). Total plant biomass was not significantly affected by a 17-day period of either aboveground or belowground herbivory alone ($F_{1,19} = 3.27$; $P = 0.086$, and $F_{1,19} = 2.45$; $P = 0.134$, respectively) and shoot-to-root biomass ratio was not affected by any of the treatments (all $P > 0.10$).

The concentrations of IGs in shoots were significantly higher in plants exposed to aboveground herbivory than in control plants ($F_{1,36} = 8.74$; $P = 0.005$; Figure 2.3a), while the concentration of IGs in roots were significantly lower in plants exposed to belowground herbivory than in control plants ($F_{1,36} = 7.23$; $P = 0.011$; Figure 2.3b). The ratio of catalpol to aucubin was not affected by any of the treatments.

Experiment 2: Effects of sequence of introduction of belowground and aboveground herbivores on aboveground herbivore performance

Weight gain of the response caterpillars was significantly reduced by aboveground herbivory ($F_{1\ 61} = 5.17$; $P = 0.027$), but only when feeding occurred before introduction of the response caterpillars (Dunnett test; $P = 0.009$) and not when feeding occurred later ($P > 0.10$) (Figure 2.4a). Belowground herbivory alone only marginally reduced weight gain of response caterpillars ($F_{1,63} = 3.79$; $P = 0.056$). The leaf area consumed by response caterpillars was significantly reduced by previous aboveground herbivory, both in the case of aboveground herbivory alone ($F_{1,61} = 6.32$; $P = 0.015$), and in combination with belowground herbivory ($F_{1,62} = 5.79$; $P = 0.019$, Figure 2.4b).
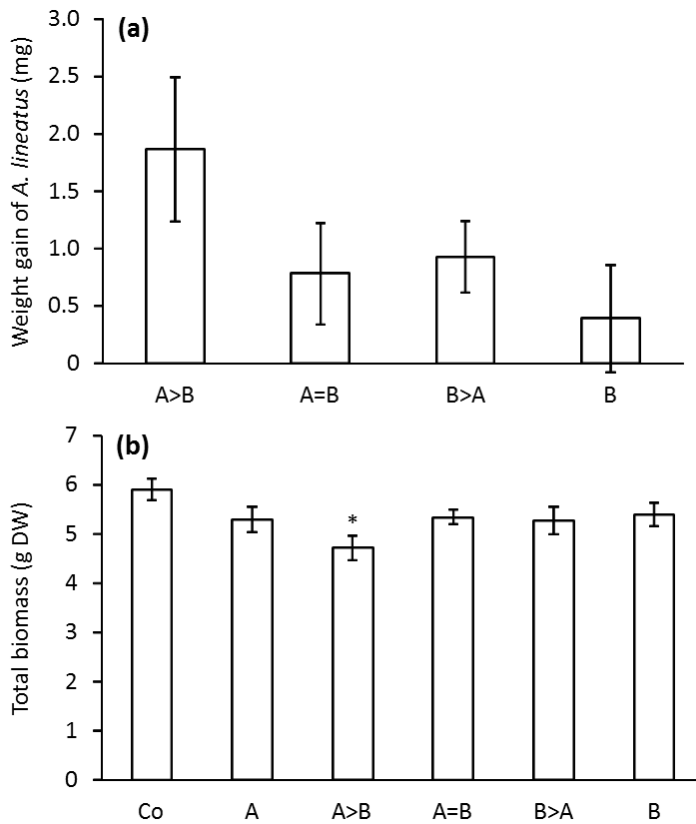
Figure 2.2 Effects of the timing of aboveground herbivore introduction on the performance of the belowground herbivore *A. lineatus* and on the host plant *P. lanceolata*. Shown are mean (± SE) (**a**) weight gain of *A. lineatus* and (**b**) total plant biomass. Plants were exposed to aboveground (A) or belowground (B) or both aboveground and belowground herbivory in different sequences (see figure 1) or left undamaged (Co). Asterisks in Figure 2.2b denote treatments significantly different from the control (Co) based on a Dunnett *post hoc* test ($P < 0.05$).

Effects of the introduced herbivores on the leaf area consumption by response caterpillars were strongly dependent on the timing of their feeding. When the two-day feeding period by *S. exigua* occurred *before* the response caterpillars were put on the plants, the leaf area consumption by response caterpillars was reduced, both in the aboveground treatment (Dunnett test; $P = 0.006$) and in the combined aboveground and belowground treatment (Dunnett test; $P = 0.011$). However, when the treatment feeding started at the same time, or after

introduction of the response caterpillars, the leaf area consumption of response caterpillars was not significantly affected (all $P > 0.10$). By contrast, belowground herbivory consistently reduced the leaf area consumption of response caterpillars ($F_{1,61} = 4.96$; $P = 0.030$), independent of the timing of their introduction (Figure 2.4b). The consumption efficiency of response caterpillars was only affected by the combined aboveground and belowground herbivory treatments ($F_{1,62} = 4.34$; $P = 0.041$; Figure 2.4c). Combined aboveground and belowground herbivory slightly increased the consumption efficiency of response caterpillars relative to that of insects on plants that were not exposed to "treatment" herbivory, but only when the treatment caterpillars started feeding prior to the response caterpillars (Dunnett test; $P = 0.045$).

Total plant biomass was only marginally reduced by the 8-day period of feeding by the response caterpillars alone compared to the control with no herbivory (Co+S vs. Co) ($F_{1,32} = 3.44$; $P = 0.073$; Figure 2.4d). However, total plant biomass was significantly reduced in combination with the aboveground herbivore treatment due to the effects of the additional 2-day feeding period by *S. exigua*, both in the absence of belowground herbivores (A+S vs. Co) ($F_{1,64} = 12.02$; $P < 0.001$) and in their presence (AB+S vs. Co) ($F_{1,64} = 7.72$; $P = 0.007$, Figure 2.4d). Root herbivory reduced root biomass ($F_{1,66} = 4.81$; $P = 0.032$), but it did not affect total plant biomass ($F_{1,66} = 2.78$; $P = 0.100$). None of the treatments significantly affected the shoot-to-root biomass ratio of the plants (all $P > 0.30$).

In the treatments involving combined effects of aboveground and belowground herbivores on response caterpillars presented above, so far we only considered the cases in which these aboveground and belowground herbivores were introduced simultaneously. Below we present results of how the sequence of their introduction further affects their impact on response caterpillars. As observed above, simultaneous introduction of aboveground and belowground herbivores *before* the response caterpillars (AB>S) reduced the weight gain of response caterpillars compared to when they were introduced at the same time as the response caterpillars (AB=S, Figure 2.5a).
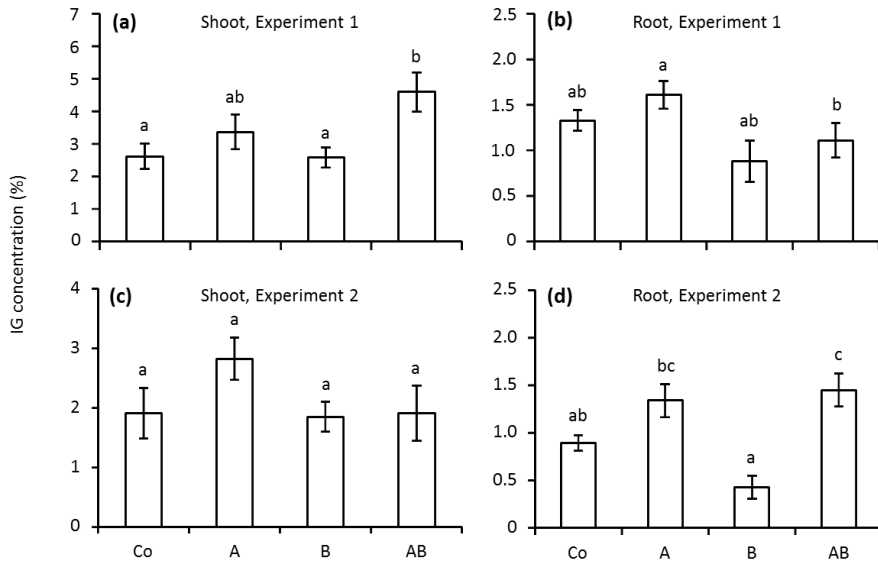
Figure 2.3 Mean (±SE) iridoid glycoside (IG) concentration (% DW) in shoot and root tissues of *P. lanceolata* exposed to no herbivory (Co), only aboveground herbivory by *S. exigua* (A), only belowground herbivory by *A. lineatus* (B), and both aboveground and belowground herbivory (AB) in experiment 1 (**a, b**) and experiment 2 (**c, d**). Plants were exposed to the herbivore treatments for 17 days in experiment 1 and for 4 days in experiment 2. For illustrative purposes, different letters have been assigned to treatments that are significantly different from each other based on a Tukey *post hoc* test following a one-way ANOVA ($P < 0.05$). For statistical analyses, see text.

This reduction was also observed when the aboveground herbivore was introduced before, but the belowground herbivore simultaneously with the response caterpillar (A>BS, Figure 2.5a), but not when the belowground herbivore was introduced before and the aboveground herbivore simultaneously with the response caterpillar (B>AS, Figure 2.5a). Statistical analysis confirmed that there was a negative effect of prior aboveground herbivory ($F_{1,58} = 6.46$; $P = 0.014$), independent of when the belowground herbivore was introduced ($F_{1,58} = 0.043$; $P = 0.84$). By contrast, effects of combined aboveground and belowground herbivory on leaf area consumption by response caterpillars were unaffected by the sequence in which aboveground and belowground herbivory was initiated (Figure 2.5b). Interestingly, the consumption efficiency of response caterpillars was reduced

when the aboveground treatment herbivore was introduced before the response herbivore, but only when the root herbivore was introduced simultaneously with the response herbivore (A>BS, Figure 2.5c) and not when the root herbivore was also introduced before the response herbivore (AB>S, Figure 2.5c) (interaction between prior aboveground and belowground herbivory: $F_{1,58}$ = 4.15; P = 0.046; Figure 2.5c). Effects on plant biomass were also independent of the onset of aboveground and belowground herbivory in the combined above- and belowground herbivory treatments (Figure 2.5d).
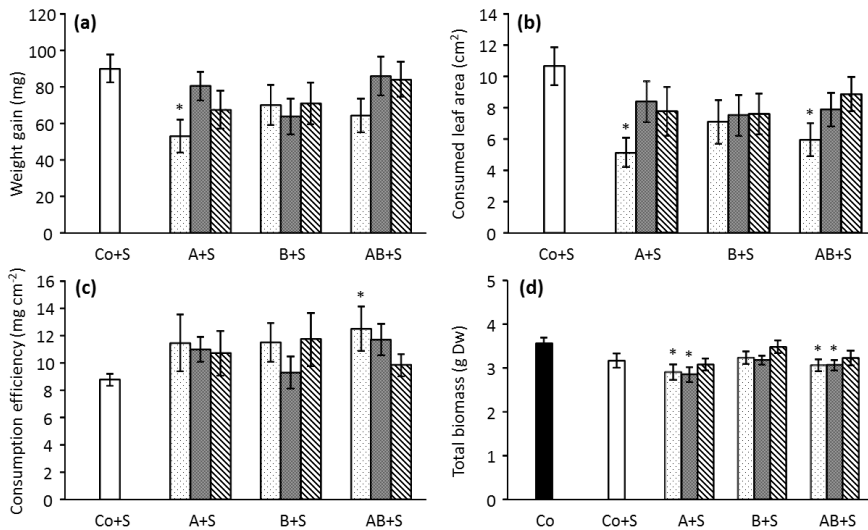

Figure 2.4 Effects of aboveground and belowground herbivores introduced before (dotted bars), at the same time as (grey bars), or after (dashed bars) the aboveground response herbivore on the performance of the response herbivore *S. exigua* and on the host plant *P. lanceolata*. Shown are mean (±SE) (**a**) weight gain, (**b**) consumed leaf area, (**c**) food consumption efficiency of *S. exigua*, and (**d**) plant total biomass. Plants were exposed to aboveground herbivory by *S. exigua* response larvae (S), and in addition to aboveground (A) or belowground (B) treatment herbivores, or not exposed to treatment herbivory (Co). Asterisks denote treatments significantly different from the control (Co+S for the herbivore traits, **a-c**; Co for the plant trait, **d**) based on a Dunnett *post hoc* test ($P < 0.05$).

Shoot IG concentrations were not significantly affected by aboveground ($F_{1,36}$ = 1.65; P = 0.208) or belowground herbivory ($F_{1,36}$ = 1.69; P = 0.20; Figure 2.3c). Root IG concentrations were significantly increased by aboveground herbivory ($F_{1,36}$ = 22.98; $P < 0.001$; Figure 2.3d), while the effects of belowground herbivory on root IG concentrations depended on aboveground

herbivory (interaction between above- and belowground herbivory; $F_{1,36}$ = 4.40; P = 0.043; Figure 2.3d). In the absence of aboveground herbivory, root herbivory decreased root IG concentrations compared to control plants. However, in the presence of aboveground herbivory, root herbivory did not affect root IG concentrations compared to aboveground herbivory alone (Figure 2.3d). Similarly, root herbivory increased the ratio of catalpol to aucubin in the roots in the absence, but not in the presence of aboveground herbivores (interaction between above- and belowground herbivory; $F_{1,36}$ = 6.12; P = 0.018).

Discussion

Our study indicates the importance of both the presence and the timing and sequence of arrival of aboveground and belowground herbivores for the performance of these organisms on their shared host plant. Importantly, we provide evidence that the timing of prior aboveground and belowground herbivory can affect the performance of later arriving aboveground herbivores. Thus, we stress the importance of considering arrival sequence in order to understand the outcome of more complex aboveground-belowground interactions.

Arrival sequence and aboveground interactions

Our study shows that the sequence in which aboveground herbivores arrive on *P. lanceolata* is an important determinant of their intraspecific interactions. Prior aboveground herbivory by *S. exigua* significantly reduced the leaf area consumption and weight gain of response caterpillars of *S. exigua*, whereas neither simultaneous arriving nor later arriving conspecifics affected the consumption or weight gain of the response caterpillars. It is unclear to what extent the induction of leaf IGs by earlier arriving conspecifics contributed to the reduced performance of the later arriving *S. exigua*. In agreement with findings from previous studies in which *P. lanceolata* was exposed to generalist (Wurst and Van der Putten 2007) and specialist (e.g. Darrow and Bowers 1999) leaf chewers, a prolonged period of aboveground herbivory (17

days, experiment 1) resulted in a significant increase in the levels of leaf aucubin and catalpol (experiment 1). However, this induction was not yet observed four days after the initiation of the two-day period of leaf herbivory in experiment 2, when the response caterpillars of *S. exigua* were introduced. Therefore, if induction or priming of IGs by previous herbivory played any roles in the reduced performance of later arriving *S. exigua*, these chemical changes were not expressed until after the response herbivores had been introduced. Alternatively, the reduced performance may have been due to the
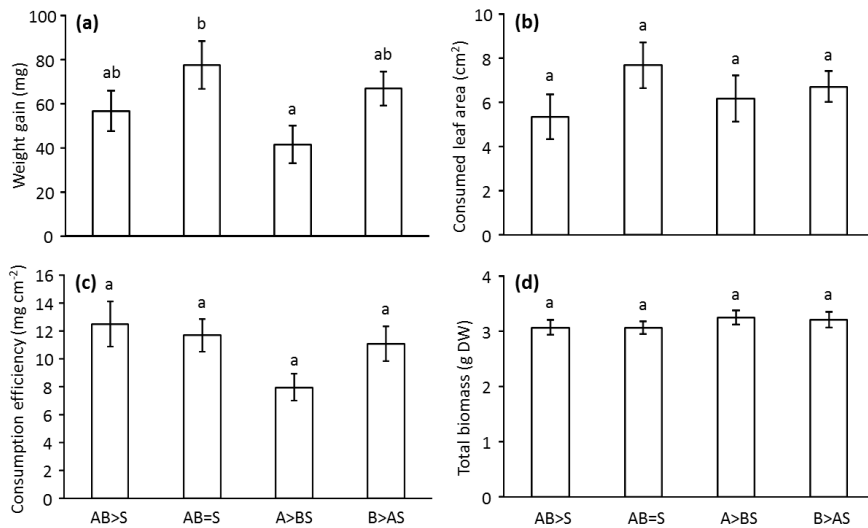

Figure 2.5 Effects of the sequence of aboveground and belowground herbivory on the performance of the aboveground response herbivore *S. exigua* and on the host plant. Shown are mean ($\pm$SE) (**a**) weight gain, (**b**) consumed leaf area, (**c**) food consumption efficiency of *S. exigua*, and (**d**) plant total biomass. Treatments represent four different introduction sequences of the aboveground (A), and belowground (B) treatment herbivores and the response herbivore (S): A and B introduced before S (AB>S), only A introduced before S (A>BS), only B introduced before S (B>AS), or all introduced simultaneously (AB=S). For illustrative purposes, different letters have been assigned to treatments that are significantly different from each other based on a Tukey *post hoc* test following a one-way ANOVA ($P < 0.05$). For statistical analyses, see text.

induction or priming of other compounds or traits than IGs. More detailed studies on the precise time pattern of induction and decay of the IG response for each of the sequences of arrival are needed to assess the role of IGs in this

response. Earlier studies using this model system have shown that such patterns can be complex (Fuchs and Bowers 2004).

Arrival sequence and aboveground-belowground interactions

In our study, the foliar generalist, *S. exigua*, did not significantly affect the performance of the root herbivore (experiment 1). On average, wireworm performance was enhanced when the aboveground herbivore was introduced prior to the wireworms and with increased feeding duration, but these effects were not significant ($P < 0.06$). The absence of a significant effect of *S. exigua* on the performance of the belowground herbivore contrasts with the pattern revealed in the meta-analysis by Johnson et al. (2012) that leaf chewing insects, when introduced prior to root feeders, generally have a negative impact on root feeding insects (see e.g. Bezemer et al. 2003; Soler et al. 2007; Erb et al. 2011). This pattern is thought to be due to the systemic induction or priming of defense compounds in the roots that takes time and requires sustained feeding of the aboveground herbivores (Erb et al. 2011, Johnson et al. 2012). In our system, however, aboveground herbivory only resulted in a transient increase in IGs in roots (four days after initiation of an 48-hour feeding bout, experiment 2), but we did not observe enhanced levels of root IGs after sustained feeding by *S. exigua* for 17 days (experiment 1). This pattern corresponds with earlier findings in this system. Induction of root IGs by aboveground herbivory was observed in a study that allowed aboveground herbivores to feed for a short period of time (72 hours) (Darrow and Bowers 1999), but not in a study in which aboveground herbivores were allowed to feed for sustained periods (Quintero and Bowers 2011). Interestingly, the transient increase in root IGs induced by *S. exigua* completely counterbalanced the reduction of root IGs caused by feeding of the wireworms. Wireworms alone strongly reduced the levels of IGs in roots and increased the ratio of the more toxic compound catalpol relative to aucubin. The latter effect has been observed for other root herbivores as well (Bennett et al. 2013). The reduction in root IG levels was initially counterbalanced by the increase in root IG levels caused by simultaneously introduced S. *exigua*. However, after sustained *S. exigua* feeding, wireworms were able to reduce root IGs even in the presence

of the aboveground herbivore. Since wireworm performance is negatively affected by root IGs (J. Huang, unpubl. results) this may be a mechanism by which wireworms can enhance their own performance. It should be noted that the absence of an effect of aboveground herbivory on the performance of the belowground herbivore in our study should be interpreted with caution. While *P. lanceolata* in temperate grasslands commonly interacts with wireworms, it may not naturally encounter *S. exigua*. The latter species was used as a model for a generalist chewing insect herbivore, but we cannot rule out that *P. lanceolata* may have evolved different responses to generalist chewers that it more often encounters in the field, resulting in a different set of consequences of such encounters for interactions with belowground herbivores.

Root herbivory by wireworms significantly reduced the leaf area consumption and marginally reduced the weight gain of the shoot herbivore *S. exigua*, independent of whether wireworms were introduced before, simultaneously with, or after the aboveground herbivore (experiment 2). Johnson et al. (2012) speculated that positive effects of root herbivores on shoot herbivores may arise if root feeders can reduce the resistance or increase the nutritional status of aboveground tissues. Wireworms did not appear to cause such effects in our host-herbivore system. Previous studies in *P. lanceolata* have shown that sustained wireworm feeding for five (Wurst and Van der Putten 2007) or eight weeks (Wurst et al. 2008) does not affect leaf nitrogen or glucose concentrations, whereas effects on leaf IGs are either absent (Wurst and Van der Putten 2007) or dependent on plant genotype (Wurst et al. 2008). Interestingly, although wireworms did not affect leaf IGs in our experiment, they did reduce leaf area consumption by the aboveground herbivore, indicating that these effects were mediated by induced plant responses other than changes in IGs.

Arrival sequence and more complex above-belowground interactions

One of the novelties of our study is that the setup also allowed us to investigate what happens in more complex above-belowground interactions.

In particular: how is the performance of aboveground response herbivores affected by the sequence of arrival of *both* conspecifics and belowground herbivores? One of the most intriguing findings was that whereas early arriving *S. exigua* were able to reduce the weight gain and consumption efficiency of their later arriving conspecifics when they also arrived prior to the root herbivores (A>BS; Figure 2.5a, c), their negative effect on consumption efficiency completely disappeared when wireworms were introduced simultaneously with the early arriving *S. exigua* (AB>S; Figure 2.5c). This suggests that wireworms either repress the induction of the defenses by *S. exigua* that are responsible for the lower consumption efficiency of their later arriving conspecifics, or that they induce compounds that compensate for the induced lower consumption efficiency. Since there were no indications that wireworms suppressed the induction of shoot IGs by *S. exigua* when they were introduced simultaneously, we speculate that this modulation may have been mediated by other compounds than IGs. Despite the alleviating effects of wireworms on the induction of traits lowering the consumption efficiency of the aboveground herbivore, later arriving *S. exigua* still suffered a lower weight gain on plants previously exposed to their conspecifics, probably due to effects of previous herbivory on other components of the relative growth rate of later arriving conspecifics. The impact of such more complex interactions between aboveground and belowground herbivores on their performance stresses the importance to get more insight in the actual patterns of the sequence and timing of arrival of above- and belowground herbivores in the field (Bezemer and Van Dam 2005). Currently we lack such information in our study system.

Effects of the interactions between aboveground and belowground herbivores on plant biomass and shoot-to-root biomass ratio in our experiments were relatively small. In experiment 2, up to eight days of wireworm feeding reduced root biomass but not total biomass, while in experiment 1, 17 days of wireworm feeding on its own did not exert significant effects on either root or total biomass, only in combination with 17 or more days of aboveground feeding by *S. exigua.* In similar experiments using this system in which wireworms were allowed to feed for five weeks (Wurst and Van der Putten

2007), wireworms reduced root biomass and induced compensatory growth of shoot tissue, whereas a feeding duration of eight weeks did not affect root biomass but enhanced shoot biomass (Wurst et al. 2008). Most probably, the feeding durations in our experiments were too short to exert such effects. Conversely, in combination with the two-day period of aboveground herbivory, aboveground response caterpillars did reduce shoot and root biomass in experiment 2, both in the presence and absence of root herbivores.

In conclusion, our study shows that the timing and sequence of appearance of aboveground and belowground herbivores can be important in mediating the outcomes of interactions between aboveground and belowground herbivores. In contrast to patterns from a meta-analysis synthesized from many different systems (Johnson et al. 2012), aboveground herbivory tended to enhance the performance of belowground herbivores when they arrived earlier, and belowground herbivory reduced leaf consumption by aboveground herbivores, irrespective of whether they arrived earlier, simultaneously or later. While our results may just reflect an exception to the general pattern, it is also possible that the predicted patterns are partly biased by the different host-enemy systems in which the different interaction sequences that formed the basis of the meta-analysis had been studied (Johnson et al. 2012). Thus, more studies are required that examine the effects of different sequences of aboveground and belowground herbivore encounters within a single system. Furthermore, our study included more complex types of sequential encounters in belowground-aboveground interactions. We showed that belowground herbivores can disrupt the induction of resistance to aboveground herbivory by prior conspecific herbivores, but only if they arrived simultaneously with the inducing aboveground herbivores. This illustrates that in a dynamic system, where aboveground herbivores may encounter prior aboveground, as well as belowground herbivory, we need to know the history of encounters in order to understand the outcomes of the above-belowground interactions.

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Chapter 3

Effects of the timing of herbivory on plant defense induction and insect performance in ribwort plantain (*Plantago lanceolata* L.) depend on plant mycorrhizal status

Minggang Wang, T. Martijn Bezemer, Wim H. van der Putten and Arjen Biere

Abstract

Plants are often exposed to antagonistic and symbiotic organisms both aboveground and belowground. Interactions between above- and belowground organisms may occur either simultaneously or sequentially, and can jointly determine plant responses to future enemies. However, little is known about time-dependency of such aboveground-belowground interactions. We examined how the timing of a 24 hr period of aboveground herbivory by *Spodoptera exigua* (1-8 days prior to later arriving conspecifics) influenced the response of *Plantago lanceolata* and the performance of later arriving conspecifics. We also examined whether these induced responses were modulated by the arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae.* The amount of leaf area consumed by later arriving herbivores decreased with time since induction by early herbivores. Mycorrhizal colonization reduced the relative growth rate (RGR) of later arriving herbivores, associated with a reduction in efficiency of conversion of ingested food rather than a reduction in relative consumption rates. In non-mycorrhizal plants, leaf concentrations of the defense compound catalpol showed a linear two-fold increase during the eight days following early herbivory. By contrast, mycorrhizal plants already had elevated levels of leaf catalpol prior to their exposure to early herbivory and did not show any further increase following herbivory. These results indicate that AMF resulted in a systemic induction, rather than priming of these defenses. AMF infection significantly reduced shoot biomass of *Plantago lanceolata*. We conclude that plant responses to future herbivores are not only influenced by exposure to prior aboveground and belowground organisms, but also by when these prior organisms arrive and interact.

Introduction

Virtually all plants in natural communities experience damage from above- and belowground organisms. In response to damage, primary and secondary metabolites and physical resistance traits often change (Karban and Baldwin 1997; Underwood 2012). Hence, via these herbivore-induced changes, the susceptibility of a plant to later arriving herbivores that feed on the plant can be altered (Thaler et al. 2002; Kaplan and Denno 2007). The impact of herbivory on later arriving herbivores depends on the specific combination of plants and attackers, and both induced resistance and induced susceptibility have been reported to occur as a response to herbivory (Koricheva et al. 2009).

In recent years, the significance of the timing of herbivory in regulating plant-herbivore interactions has been increasingly recognized (Blossey and Hunt-Joshi 2003; Nykänen and Koricheva 2004; Sullivan and Howe 2009; Erb et al. 2011; Johnson et al. 2012). The time lag between damage and the onset of defense, as well as between cessation of damage and the relaxation of defense are crucial in determining the establishment or feeding of later arriving herbivores (Karban 2011). Both lags may depend on the plant species maintaining the induced defense, but also on the timing of herbivory in relation to plant ontogeny (Young et al. 2003; Boege and Marquis 2005; Gomez et al. 2010; Wang et al. 2014). Generally, younger plants are easier to be induced and their induced defenses show more plastic responses to other biotic or abiotic factors while older plants that take more time to induce defenses typically maintain these induced defenses for a longer time (Fuchs and Bowers 2004).

Plant-induced responses to herbivory are not restricted to locally damaged organs, but can also be systemically expressed in undamaged tissues (Van Dam et al. 2004; Bezemer and Van Dam 2005). Several studies have shown that plant responses to herbivores can be altered by the plant's interaction with belowground microbial plant symbionts such as mycorrhizal fungi and plant growth promoting rhizobacteria (Pozo and Azcón-Aguilar 2007; Van Oosten et al. 2008; Pineda et al. 2010; Zamioudis and Pieterse 2012; Pangesti

et al. 2013). Mycorrhizal fungi are root-associated organisms that can influence a plant's response to herbivory via a diversity of mechanisms. The nutritional status, level of secondary metabolites, and tolerance to abiotic and biotic stress of a plant can all be altered by interactions between the plant and mycorrhizal fungi (Smith and Read 2008). This subsequently can alter the plant responses to its herbivores. These mycorrhizae-induced changes in the plant can be either beneficial or detrimental for herbivores that feed on the plant, and the strength and direction of these effects may depend on the feeding mode or specialization of the herbivore (Bennett et al. 2006; Koricheva et al. 2009; Borowicz 2013). A meta-analysis of 34 studies showed that arbuscular mycorrhizal fungi (AMF) predominantly have negative effects on the performance of generalist chewing herbivores, but that they can enhance the performance of specialist chewing herbivores (Koricheva et al. 2009). Plant secondary compounds are often toxic for generalists, but can be used as feeding stimulants by specialist chewers (Giamoustaris and Mithen 1995; Agrawal 2003). Hence, changes in the production of these chemicals have been proposed as a mechanism by which AMF modulate interactions between a plant and its herbivores (Bennett et al. 2009; De Deyn et al. 2009). AMF can modulate shoot levels of secondary metabolites in two ways. First, AMF can simply induce defense metabolites in shoots. Secondly, mycorrhizal infection can modulate the plant's ability to respond to herbivores, causing a stronger or faster increase in the concentration of defense chemicals in the shoots in response to herbivory (Jung et al. 2012; Song et al. 2013), a phenomenon known as defense priming (Conrath et al. 2006; Pozo and Azcón-Aguilar 2007).

So far, we are not aware of any studies that have explored how AMF interfere with the timing of induction following herbivory and its consequences for the performance of later arriving herbivores. In the current study, we tested whether and how mycorrhization influences the time course of induction of plant defense compounds and how it affects the performance of later arriving herbivores. To examine how mycorrhization interacts with timing of herbivory we exposed mycorrhizal and non-mycorrhizal *Plantago lanceolata* plants to controlled levels of herbivory at different times prior to introducing response herbivores.

Plantago lanceolata L. (Plantaginaceae) (ribwort plantain) is a short-lived perennial forb with a worldwide distribution. It can associate with a multitude of species of AM fungi in the field (Johnson et al. 2004) and is frequently employed as a model system in studies of plant-mycorrhiza interactions (e.g. Bennett et al. 2009). *P. lanceolata* produces several classes of secondary metabolites that can be induced by herbivory (Sutter and Müller 2011). An important class are the iridoid glycosides (IGs), whose levels (mainly aucubin and catalpol) can constitute up to more than 10% of leaf dry weight (Bowers et al. 1992). These compounds are toxic or deterrent to non-adapted generalist herbivores (Bowers and Puttick 1988; Bowers and Stamp 1992; Darrow and Bowers 1999; Harvey et al. 2005; Reudler et al. 2011) but serve as feeding or oviposition cues for specialists (e.g. Nieminen et al. 2003; Reudler et al. 2008). We chose to focus on these compounds since their tissue levels in *P. lanceolata* are known to be influenced by both herbivory (e.g. Fuchs and Bowers 2004) and by colonization with AM fungi (e.g. Bennett et al. 2009). The induction of IGs by herbivores depends e.g. on the ontogeny of the plant (Quintero and Bowers 2011, 2012) and on the time lag between induction and response (Fuchs and Bowers 2004). Strength and direction of induction of IGs by mycorrhizae in *P. lanceolata* strongly depends on AMF species (Bennett et al. 2009) and varies among studies (e.g. Gange and West 1994, Fontana et al. 2009, Schweiger et al. 2014). Mycorrhization of *P. lanceolata* has been shown to suppress plant induced responses to aboveground herbivory and to alter the proportion of catalpol in the total IG level following herbivory (Bennett et al. 2009; Bennett et al. 2013).

As AMF species we used *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (Glomeraceae) (formerly *Glomus mosseae). F. mosseae* forms a symbiotic relationship with many plant species including *P. lanceolata* (Karasawa et al. 2012; Orlowska et al. 2012). It has been used in previous studies on plant-mediated AMF-herbivore interactions in other systems, both at the phenotypic (Borowicz 2013) and molecular level (Song et al. 2013, Fernandez et al. 2014). As representative of an aboveground generalist chewing herbivorous insect we used the southern beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), that attacks a wide range of plant

species (Greenberg et al. 2001). It originates from Southeast Asia but nowadays has a global distribution. The larvae go through five instars during development and can produce several generations per year.

We tested three hypotheses: i) Plants need time to activate induced defense and the induced defense decays over time. Hence, early or late timing of prior damage relative to the arrival of later herbivores will result in lower levels of induced defense compounds (IGs) and in a better performance of these herbivores than when prior damage occurs at intermediate time point. ii) AM fungi will prime the plant for a quicker or stronger response to herbivory. Therefore, we expect that AMF colonization will either strengthen the induced plant response (IGs level) to later arriving herbivores, or result in a more rapid response, shifting the onset of the response to an earlier time point. iii) AMF colonization will increase shoot biomass and reduce negative effects of previous herbivory on shoot biomass.

Materials and Methods

Plants, herbivores and AMF

 Seeds of *P. lanceolata* were obtained from a full-sib cross between two parents originating from a hayfield and a pasture in the Netherlands, respectively. The seeds were surface sterilized using 1% sodium hypochlorite (sterilized for 1 min followed by 4 times 5 min rinsing with demineralized water), sown on glass beads and placed in an incubator (16/8 h light and 25/20 ℃ day/night) until seedling emergence.

Eggs of *S. exigua* were obtained from the Laboratory of Entomology, Wageningen University, The Netherlands. After hatching larvae were reared on artificial diet (Biere et al. 2004) in a growth chamber at 22 ℃, 16:8 h light/dark photoperiod and at 70% RH.

F. mosseae inoculum was purchased from Symbiom Ltd. (Lanskroun, Czech Republic) (Strain BEG 198).

Experimental set-up

 Soil was collected from a restoration grassland (De Mossel, Ede, The Netherlands) where *P. lanceolata* is abundant. In the laboratory, the soil was sieved through a 0.5 cm mesh, homogenized and gamma-sterilized (>25 KGray). The sandy-loam mineral soil was mixed with sterilized sorbix (Damolin, Fur, Denmark) and sand in a 1:2:2 (soil : sand : sorbix, vv^{-1}) proportion to promote drainage. A total of 206 pots (9×9×10 cm) were filled with 600 g of soil-sand-sorbix mixture. The pots were then watered with 50 ml of a soil microbial wash extracted from 25 kg fresh soil suspended in 25 L tap water and filtered through 75, 45 and 20 μm filters to obtain a microbial wash that excluded AMF propagules. The microbial wash was added to establish a background microbial community in the sterile substrate mixture. Thereafter, 110 pots were inoculated with 12 g vital *F. mosseae* inoculum (Mycorrhizal plants, M) that had been mixed with 0.5 g sterilized bonemeal (16% phosphate, Ecostyle, The Netherlands) and 7.5 g fully mixed sterile soil-sand-sorbix mixture. Bonemeal was used as a slow-release source of phosphorus in the experiment to promote mycorrhizal performance. Its addition resulted in 133 mg of total P per kg of soil, corresponding to ca. 4 mg of water-soluble P per kg of soil (Ylivainio et al. 2008). The other 96 pots were inoculated with 12 g autoclaved (30 min at 121°C) *F. mosseae* inoculum mixed with 0.5 g sterilized bonemeal (Ecostyle, The Netherlands) and 7.5 g sterile soil-sand-sorbix mixture (Non-mycorrhizal plants, NM). One seedling was then planted into each pot and pots were watered three times per week (two times using demineralized water and one time using 50 ml of a 0.5 strength Hoagland solution without phosphate). Five weeks after transplantation, the number of main rosette leaves on each plant was counted and the length of each leaf was measured. Plant size was determined by calculating the total leaf length of each plant to enable an equal initial plant size distribution for each treatment when allocating plants to the different timing and herbivore treatments within the mycorrhizal and non-mycorrhizal groups (see below).

The experiment was set up to examine the effects of mycorrhizal infection, aboveground herbivory and the timing of aboveground herbivory on the

performance of a later feeding aboveground herbivore and on induced plant defense. To standardize the amount of damage caused by the 'treatment' herbivores, two clip-cages (2 cm diameter), each with one fourth instar *S. exigua* larva were simultaneously placed on the distal part of the seventh youngest fully expanded mature leaf for 24 h. During this time two areas of 3.14 cm^2 were consumed. The herbivory treatment was initiated at five different times: 8 days, 4 days, 2 days, and 1 day before the introduction of response herbivore (see below). The 8-day-treatment was initiated five weeks after transplantation. Empty clip-cages were put on subsets of the (no-herbivory) control plants at 8, 4, 2, and 1 days before introduction of response caterpillars. The experiment followed a full factorial design with 2 AMF treatments (M = mycorrhizal, NM = non-mycorrizal) and 5 herbivory treatments (8 d, 4 d, 2 d, 1 d, control = no treatment herbivory). Of the 206 plants in total, 135 plants (15 replicates for herbivory treatments and 12 replicates for control within M plants; 13 replicates for herbivory treatments and 11 replicates for controls within NM plants) were used to examine the effects of mycorrhizal presence, herbivory and timing of herbivory on subsequent herbivore performance and induced plant defense. Effects on subsequent herbivores were tested in two bioassays described below. The 71 remaining plants (8 M and 7 NM replicates for each of the four herbivory treatments, and 6 M and 5 NM replicates for their respective controls) were not subjected to any bioassay but used to assess plant biomass production as a function of AMF and induction by treatment caterpillars.

Bioassays

The effects of AMF association, previous herbivory and timing of previous herbivory on later arriving herbivores were examined using two bioassays.

Detached-leaf bioassay For this bioassay, two leaves (the fifth and sixth youngest true leaf) of each of the 135 bioassay plants (leaf number: mean = 9.94; SE = 0.07) were excised and weighed at t=0 (8, 4, 2 and 1 days after the respective 24 hr herbivory treatments, 43 days after seedling transplantation). From each leaf, 3 leaf disks were taken around the mid-vein using a sharp cork borer (16

mm diameter) so that a total of 6 leaf cuttings were obtained from each plant (Biere et al. 2004). Two of these six leaf disks (one from each leaf) were used to determine fresh weight and dry weight (after drying for 72 hrs at 50 ºC). The four remaining disks were placed on moist filter paper in a Petri dish (9 cm in diameter) where a freshly-moulted pre-weighed 3rd instar *S. exigua* larva ("bioassay" or response caterpillar, mean = 9.66 mg; SE = 0.07) was introduced. The Petri dishes were placed in a growth chamber at 25 ºC and a photoperiod of 16/8 h (light /dark). After exactly 24 h, the larvae were removed and immediately reweighed and then frozen (-20 ºC). The frozen caterpillars were oven-dried at 50 ºC and their dry weight was determined. The remaining, non-consumed material of the leaf disks was collected and scanned using a photo scanner (EPSON, PERFECTION 4990, Japan) to determine the leaf area consumed by the bioassay caterpillar using the software WinFOLIA (Regent Instruments, Sainte-Foy, Canada). The remaining leaf disks were then oven-dried at 50 ℃ to enable estimation of the leaf dry weight consumed by bioassay caterpillars. The remaining plant material of the two leaves from which leaf disks were taken was oven-dried (50 ºC) and used for chemical analysis (see below). Detached leaf 24-hr bioassays have been successfully applied in this system before (Biere et al. 2004) and have shown good correlations between IG concentrations of leaves at the time of detachment and *S. exigua* performance on detached leaves. This indicates that even though absolute levels of primary or secondary metabolites may differ between attached and detached leaves, the latter are still likely to represent relevant differences in leaf chemical quality between the plants in the experiment.

Whole plant bioassay After excision of two leaves from the 135 plants used for the detached leaf bioassay at day 0, these plants were individually caged using cylindrical mesh cages (height 1 m, diameter 35 cm) for the second bioassay, assessing their responses to previous herbivory in the longer term (8 days extra, see below). One pre-weighed 3rd instar *S. exigua* (mean = 24.0 mg; SE = 0.32) was then introduced into each of the 135 cages with bioassay plants. The larvae could move freely within the cage. Eight days later, the surviving caterpillars were collected, reweighed and oven-dried. Mortality was high in the cages and dead larvae were also collected and oven-dried.

After collection of the caterpillars, the 135 bioassay plants were harvested. All leaves of each plant were scanned and the leaf area consumed by the response caterpillars was determined using the same equipment and software as described above for the detached leaf bioassay. Roots were carefully removed from the soil and rinsed. A small sample of the roots was taken from nine randomly selected mycorrhizal and five non-mycorrhizal plants that had not been subjected to previous herbivory to quantify the extent of root colonization by *F. mosseae*. Leaf and root material was then oven-dried (50 °C) and dry weight was determined. The 71 plants not used in the bioassays were caged as well and harvested simultaneously with the bioassay plants.

Iridoid glycoside analysis

All 135 leaf samples of the bioassay plants were weighed and ground. Twenty-five mg of each sample was extracted overnight in 70% methanol, and then filtered (12-15 µm) followed by a dilution of 10 times with ultrapure water. The concentrations of the IGs aucubin and catalpol were analysed using HPLC as described by Marak et al. (2002b).

Caterpillar performance

For the detached-leaf bioassay, three indices were calculated to characterize herbivore performance following Waldbauer (1968). Relative growth rate of caterpillars was calculated as RGR = (CDW2-CDW1) / (0.5*(CDW1+CDW2)), where CDW1 and CDW2 are initial and final (after 24 hr) dry weight of caterpillars and CDW1 of each caterpillar was estimated from its initial fresh weight and its final fresh:dry weight ratio. The relative consumption rate of caterpillars was calculated as RCR = (LDW2-LDW1) / (0.5*(CDW1+CDW2)), where LDW1 and LDW2 are initial and final dry weight of the four leaf disks and LDW1 for each plant was calculated from the initial fresh weight of the four leaf disks and the initial fresh:dry weight ratio of the two leaf disks from the corresponding plant. The efficiency of conversion of ingested food (ECI) was calculated as (CDW2-CDW1) / (LDW2-LDW1).

Plant size and biomass

 Number of rosette leaves and maximum leaf length of all 206 plants were used to analyse effects of AMF on plant size five weeks after transplantation, prior to herbivore treatments. Plant biomass of the 71 plants not used in the bioassays was used to analyse effects of AMF and induction by 24 hrs of feeding by treatment caterpillars on plant dry weight production in the absence of caterpillar feeding seven weeks after transplantation. Leaf mass was corrected for the dry weight of the two leaf discs that were removed for the early herbivory treatment, estimated based on the area:dry weight ratio of the excised leaf discs for the bioassay of the corresponding plant. Leaf and root biomass of the 135 bioassay plants was used to analyse the effects of AMF and induction by treatment caterpillars on plant dry weight production in the presence of caterpillars feeding for an eight-day period. Leaf dry weight of these plants was corrected by adding the dry weight of the two excised leaves based on the fresh weight:dry weight ratio of the remainder of these leaves after removing the six leaf disks. Root dry weights of the plants from which subsamples were used for examining mycorrhizae were corrected by adding the dry weight of these subsamples (estimated on the basis of the the root fresh:dry weight ratio for the corresponding plants) to the root dry weights of these plants.

Root colonization by AMF

Colonization of roots by *F. mosseae* was quantified using the gridline intersect method (McGonigle et al. 1990). Briefly, at least 100 small root pieces per root sample were cleared in 10% KOH for 10 min at 95 °C, and stained with a mixture of vinegar (5% acetic acid) and 5% Scheaffer black ink for 8 min at 80-90 °C. Stained roots were mounted on slides and checked for confirmation of mycorrhizal colonization of plants in the mycorrhizal treatment and absence of mycorrhizal colonization in control plants (Vierheilig et al. 1998) under a compound microscope (BH-2; Olympus, Tokyo, Japan) at ×40 magnification. The presence of AMF structures (hyphae, arbuscules, vesicles or spores) was

scored at 120 grid intersections per root sample and the scores were averaged per plant.

Statistical analysis

 To determine how AMF, previous herbivory (the 24 hr period of feeding by treatment caterpillars) and the timing of previous herbivory affected the performance of response (bioassay) caterpillars and leaf IG concentrations in the detached leaf bioassay, we performed three-way *ANOVA*s in which AMF status (presence or absence of mycorrhizal fungi) and previous herbivory (presence or absence of treatment herbivory) were used as categorical factors and the timing of previous herbivory (or an empty clip cage), 8, 4, 2, 1 day before introduction of bioassay caterpillars, was included as a continuous variable. Due to high mortality of caterpillars in the whole plant bioassay, possibly partly caused by pathogen infestation, no attempts were made to analyse caterpillar performance for this bioassay. Instead, differences in survival between AMF and non-AMF plants were analysed using generalized linear models with a binomial distribution and logit link function.

To determine the effects of AMF and induction by a 24 hr period of caterpillar feeding (previous herbivory) on shoot and root biomass of the 71 plants that were not used in the bioassays, we used a two-way *ANOVA* with AMF (presence or absence) and previous herbivory (herbivory at 8d, 4d, 2d, 1d before introduction of bioassay caterpillars and no herbivory) as fixed factors. The data did not allow a full three-way analysis with AMF, previous herbivory and timing of previous herbivory, since there were only one or two replicates for the no-herbivory control (empty clip-cage) treatment per time point for these 71 plants. Instead, the replicates within the no-herbivory treatment for each time point were grouped together as one level ("no previous herbivory") of the factor previous herbivory. Note that effects of previous herbivory in this analysis are indicative of costs of induction rather than costs of leaf removal since the leaf area that was removed by treatment caterpillars from induced plants was added to the leaf biomass. Similarly we tested effects of AMF and previous herbivory (8d, 4d, 2d, 1d before the

introduction of bioassay caterpillars and no herbivory) on root and shoot biomass of the 135 plants that were used in the bioassays. For all data the residuals were checked for normality using a Kolmogorov-Smirnov one-sample test and for homogeneity of variance using a Levene test before analysis and transformed when necessary.

Results

Effects of AMF and previous herbivory on plant biomass

AMF did not affect plant size, measured as total leaf length, at the age of five weeks, just prior to the herbivory treatments ($F_{1,204}$ = 2.16, P = 0.144). However, AMF had minor effects on plant morphology. Specifically, AMF plants produced a slightly larger number of main rosette leaves (10.2 vs. 9.9, $F_{1,204}$ = 5.96, P = 0.015) at the expense of a slightly smaller maximum leaf length (20.0 vs 20.6 cm, $F_{1,204}$ = 4.60, P = 0.033). However, at the age of 7 weeks, AMF had significantly reduced the shoot biomass of the plants, both the ones that had not been used for the bioassays (by on average 7.1 %, Table 3.1, P < 0.001, Figure 3.1a) and the ones that had been used for the bioassays (by on average 6.8%, Table 3.1, P < 0.001; Figure 3.1b). Induction of plants by treatment caterpillars did not significantly affect the shoot biomass of these plants (Table 3.1, Figure 3.1a). Root biomass was not affected by either AMF or previous herbivory (Table 3.1, Figure 3.1c). A similar pattern was observed for the plants that had been exposed to an eight-day period of feeding by later arriving herbivores except that AMF also had a negative effect on root biomass. On these plants, AMF reduced the shoot and root biomass by on average 6.8% (Table 3.1, P < 0.001, Figure 3.1b) and 7.4%, respectively (Table 3.1, Figure 3.1d). There were no effects of induction of plants by previous herbivory on shoot or root biomass, nor any interactions between AMF and previous herbivory (Table 3.1). Similar results were obtained when we specifically tested the contrast between "no herbivory" (empty clip cage plants) and "previous herbivory" (all other levels of this factor, i.e. previous herbivory at 1, 2, 4, and 8 days before introduction of bioassay caterpillars combined) and its interaction with AMF (all P > 0.09).

Table 3.1 ANOVA results for impacts of AMF inoculation and previous herbivory on the shoot and root biomass of *P. lanceolata* in the absence or presence of an eight-day feeding period by later arriving herbivores (LAH)

		Non-bioassay (-LAH)					Bioassay (+LAH)			
		Shoot mass		Root mass			Shoot mass		Root mass	
	df1	F	p	F	p	df2	F	p	F	p
AMF (M)[a]	1	**11.06**[c]	**0.001**[c]	0.02	0.884	1	**23.37**[c]	**<0.001**[c]	**7.42**[c]	**0.007**[c]
Herbivory[b] **(H)**	4	0.82	0.520	1.24	0.303	4	1.29	0.276	0.74	0.570
M*H	4	0.77	0.550	0.54	0.710	4	0.82	0.512	1.08	0.369
Error	61					125				

[a]AMF inoculation indicates two treatment groups with vital or autoclaved sterile AMF.
[b]Herbivory refers to treatments within non-AMF or AMF groups that were exposed to previous herbivory 1, 2, 4 and 8 days prior to the introduction of bioassay caterpillars or no herbivory.
[c]Bold values indicate significant effects at $p < 0.05$.

Effect of AMF, previous herbivory and timing of previous herbivory on caterpillar performance

Roots of plants from the mycorrhizal treatment that had not experienced herbivory showed low but consistent levels of colonization by AMF structures (hyphae, arbuscules and vesicles, 22.9 ± 2.5%, n = 9). In the control treatment, no *F. mosseae* structures were found (n = 5).

Detached-leaf bioassay AMF colonization significantly reduced the relative growth rate (RGR) of bioassay caterpillars in the detached-leaf bioassay ($F_{1, 127}$ = 4.45, $P = 0.037$, Table 3.2, Figure 3.2a). Differences in RGR among caterpillars could be mainly explained by variation in the efficiency with which they converted the ingested food into biomass (ECI, explaining 85.3% of variation), whereas variation in their relative consumption rates (RCR) explained very little variation in RGR (1.5%). Although this suggests that AMF reduced food quality rather than intake rates, effects of AMF on neither of these two individual components of RGR were statistically significant (Table 3.2, Figure 3.2b, c). In accordance with the negligible contribution of RCR to differences in RGR, the negative effect of AMF on caterpillar RGR was not reflected in a reduced rate of leaf area consumption (Table 3.2, Figure 3.2d). Previous herbivory did not have a significant main effect on leaf area consumption by bioassay caterpillars (Table 3.2). However, interestingly, the effect of previous

herbivory on leaf area consumption significantly increased over time (Table 3.2, *P* = 0.04), from no reduction observed when herbivory occurred one day earlier, to 13 and 14% reduction in leaf area consumption when herbivory occurred eight days earlier, for non-mycorrhizal and mycorrhizal plants, respectively. AMF did not interact with the plant's response to previous herbivory (no AMF × herbivory, nor AMF × herbivory × time interactions, Table 3.2) in terms of leaf area consumption by bioassay caterpillars or their RGR.
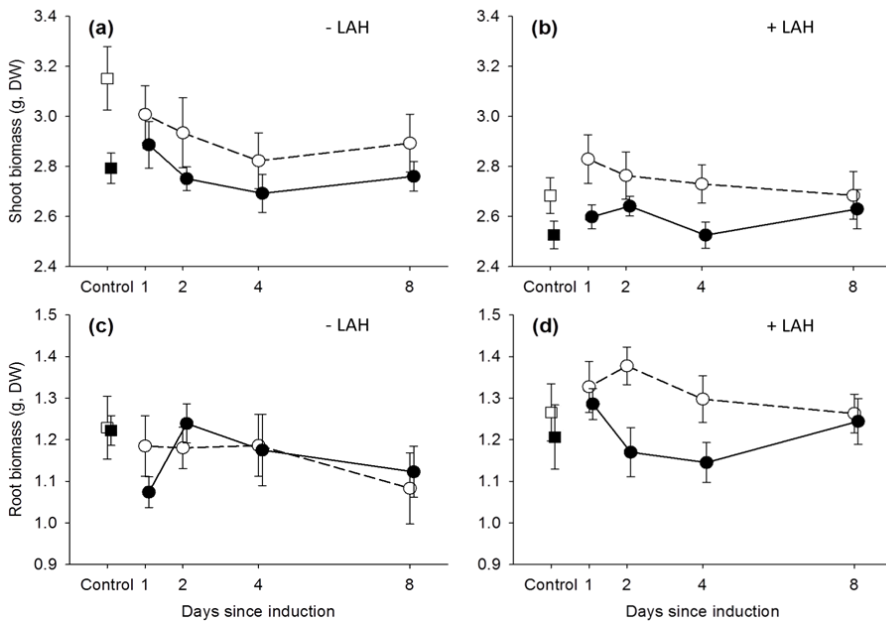

Figure 3.1 Mean (±SE) shoot (**a, b**) and root (**c, d**) biomass of mycorrhizal (filled symbols) and non-mycorrhizal (open symbols) *P. lanceolata* plants that were (circles) or were not (squares) exposed to previous herbivory 1, 2, 4, and 8 days prior to the introduction of later arriving herbivores (LAH) and that were (+LAH) or were not (-LAH) exposed to an eight-day period of feeding by later arriving herbivores prior to harvest. Filled symbols: n=15 for +LAH and n=8 for -LAH; open symbols: n=13 for +LAH and n=7 for -LAH. See table 3.1 for statistics. Note: the controlled amount of leaf biomass removed by the previous treatment herbivores has been added to the shoot biomass.

Whole-plant bioassay Bioassay caterpillars in the whole-plant bioassay (that fed on caged plants for eight days) suffered unexpectedly high levels of mortality (57.8%), which precluded further analysis of effects of AMF and previous

herbivory on their performance. Survival rates were significantly higher on mycorrhizal plants (50.0%) than on non-mycorrhizal plants (33.3%) (*Wald* = 7.93, *P* < 0.005), both on plants that had experienced previous herbivory (60.0 vs. 32.7%) and plants that had not (75.0 vs. 36.4%). There was no significant effect of previous herbivory on survival (*Wald* = 2.41, *P* = 0.12), nor an interaction between AMF and previous herbivory (*Wald* = 3.01, *P* = 0.08).

Table 3.2 ANOVA results for effects of AMF inoculation, previous herbivory and timing of induction on relative growth rate (RGR), relative consumption rates (RCR), efficiency of conversion of ingested food and consumed leaf area (CLA) of bioassay caterpillars

		RGR		**RCR**		**ECI**		**CLA (cm²)**	
	df	F	*p*	F	*p*	F	*p*	F	*p*
AMF (M)[a]	1	**4.45**[d]	**0.037**[d]	1.32	0.253	2.69	0.104	0.27	0.062
Herbivory (H)[b]	1	1.10	0.295	0.46	0.498	0.47	0.496	0.73	0.396
Time (T)[c]	1	0.06	0.815	0.00	0.960	0.05	0.816	**4.32**[d]	**0.040**[d]
M*H	1	0.32	0.574	0.93	0.337	0.00	0.992	0.39	0.534
M*T	1	2.37	0.127	3.77	0.054	0.49	0.487	0.99	0.321
H*T	1	1.91	0.170	0.83	0.365	0.91	0.342	0.13	0.717
M*H*T	1	0.38	0.541	1.94	0.167	0.02	0.890	0.69	0.407
Error	127								

[a]AMF inoculation indicates two treatment groups with vital or autoclaved sterile AMF.

[b]Herbivory refers to plants that were or were not exposed to a 24 hr period of herbivory prior to introduction of bioassay caterpillars.

[c]Time refers to when plants assigned to previous herbivory treatments were exposed to herbivory or empty clip cages (8, 4, 2, and 1 day before the introduction of bioassay caterpillars).

[d]Bold values indicate significant effects at *p* < 0.05.

Effects of AMF, previous herbivory and timing of previous herbivory on shoot IG concentration

Overall, AMF colonization of plant roots increased the shoot concentration of catalpol ($F_{1, 127}$ = 7.17, *P* = 0.008, Figure 3.3a, Table 3.3), whereas the increase in the shoot concentration of aucubin was not significant ($F_{1, 127}$ = 3.43, *P* = 0.066, Figure 3.3b, Table 3.3). Neither herbivory nor the timing of herbivory had a significant effect on shoot concentrations of aucubin or catalpol (Table 3.3). However, when we specifically focus on the plants that had been subjected to previous herbivory, an interesting pattern arises. In non-mycorrhizal plants, the concentration of catalpol significantly increased ca. two-fold over the time period between one and eight days following exposure to herbivory (linear

regression, $F_{1,50}$ = 8.02, P = 0.007). By contrast, mycorrhizal plants, that had already 64% higher leaf catalpol concentrations at the start of the herbivory treatment (Figure 3.3b, squares, $F_{1,26}$ = 5.10, P = 0.033), did not show a further increase following herbivory (linear regression, $F_{1,58}$ = 0.07, P = 0.796), resulting in a significant interaction between presence or absence of AMF and timing of previous herbivory for plants exposed to herbivory ($F_{1,108}$ = 5.56, P = 0.020). No such effects were observed for aucubin.
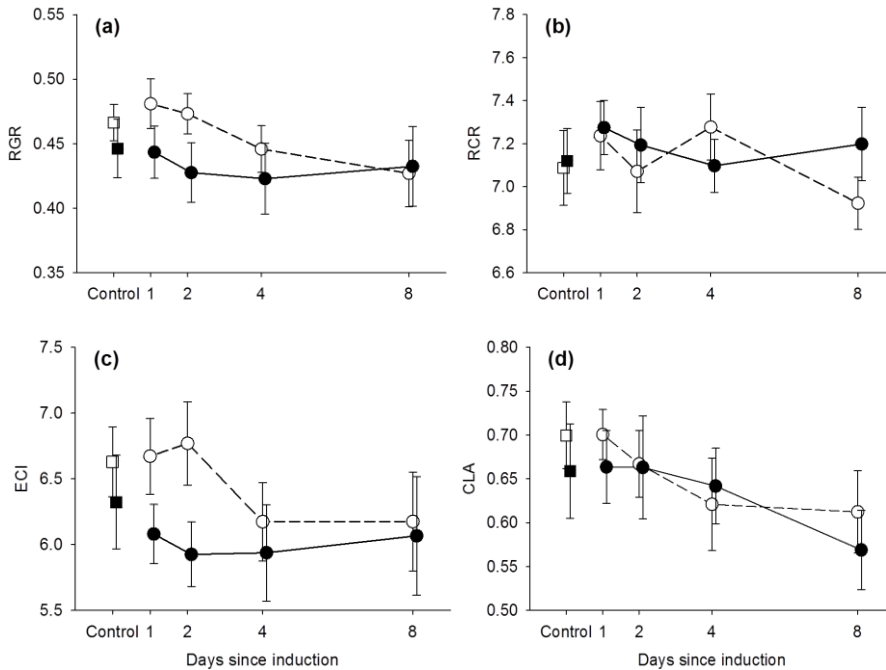

Figure 3.2 Mean (± SE) Relative growth rate (RGR, **a**), relative consumption rate (RCR, **b**), efficiency of conversion of ingested food (ECI, **c**), and consumed leaf area (CLA, **d**) of bioassay caterpillars after 24 h of feeding on excised leaves of mycorrhizal (filled symbols, n=15) and non-mycorrhizal (open symbols, n=13) *P. lanceolata* plants. Plants had either been exposed to no herbivory (Control, squares), or to a controlled 24 hr period of herbivory 1, 2, 4, or 8 days prior to the bioassay (circles). See table 3.2 for statistics.

Table 3.3 ANOVA results for impacts of AMF inoculation, previous herbivory and timing of induction on the concentration of aucubin and catalpol in leaves of *P. lanceolata* in the absence of later herbivore feeding

		Aucubin		Catalpol	
	df	F	*p*	F	*p*
AMF (M)[a]	1	3.43	0.066	**7.17**[d]	**0.008**[d]
Herbivory (H)[b]	1	0.15	0.699	0.15	0.695
Time (T)[c]	1	0.66	0.419	2.49	0.117
M * H	1	2.10	0.150	0.18	0.672
M* T	1	1.06	0.305	1.79	0.183
H * T	1	0.01	0.925	0.01	0.926
M * H * T	1	0.22	0.637	0.19	0.665
Error	127				

[a]AMF inoculation indicates two treatment groups with vital or autoclaved sterile AMF.

[b]Herbivory refers to plants that were or were not exposed to a 24 hr period of herbivory prior to introduction of bioassay caterpillars.

[c]Time refers to when plants assigned to previous herbivory treatments were exposed to herbivory or empty clip cages (8, 4, 2, and 1 day before the introduction of bioassay caterpillars).

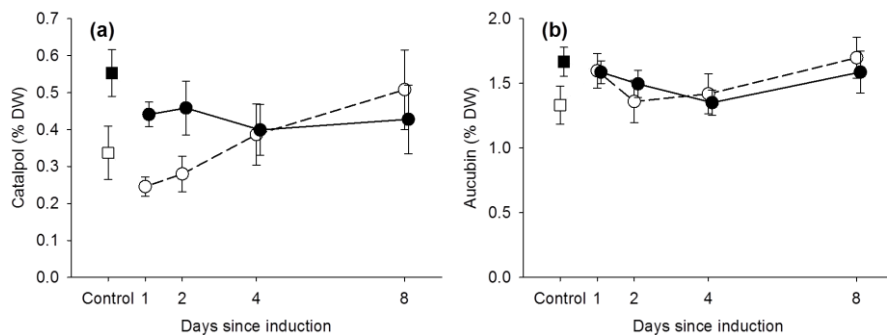[d]Bold values indicate significant effects at $p < 0.05$.

Figure 3.3 Mean (±SE) shoot catalpol (**a**) and aucubin (**b**) concentration of mycorrhizal (filled symbols, n=15) and non-mycorrhizal (open symbols, n=13) *P. lanceolata* plants that had experienced no herbivory (Control, squares) or a controlled 24 hr period of herbivory 1, 2, 4, or 8 days prior to the bioassay (circles). See table 3.3 for statistics.

Discussion

Our study of interactions between the host plant *P. lanceolata,* the arbuscular mycorrhizal fungus *F. mosseae,* and the foliar insect herbivore *S. exigua* shows that root colonization by the fungus (1) reduces the shoot biomass of the host

plant, (2) systemically induces a defense metabolite (catalpol) in the shoots of the host plant, (3) alters the time course of induction of this defense metabolite by the host plant in response to foliar insect herbivory, and (4) reduces the relative growth rate of later arriving conspecific foliar insect herbivores.

Effects of AMF and shoot herbivory on plant biomass

In contrast to our hypotheses, induction of plants by *S. exigua* did not reduce shoot biomass, neither in the presence, nor in the absence of AMF. Since similar, very small, amounts of leaf tissue were removed from plants exposed to the 24-hr period of previous herbivory and from control plants, the absence of effects of previous herbivory on shoot biomass indicates that there were no costs of induction, rather than no costs of leaf removal. Such costs may be small under the no-competition and relatively high resource conditions as in our experiment (Cipollini et al. 2003). In our study, shoot biomass of mycorrhizal plants was lower than that of non-mycorrhizal plants, independent of whether the plants were exposed to previous herbivory or not (no interaction between previous herbivory and AMF). It has long been recognized that AMF can not only have positive effects on host plant growth, but can also negatively affect plant growth under a large set of environmental conditions (Johnson et al. 1997; Klironomos 2003). Our results corroborate previous studies in *P. lanceolata* showing a continuum of mycorrhizal growth responses (the difference in biomass between mycorrhizal and non-mycorrhizal plants weighted by that of non-mycorrhizal plants) from positive or neutral (e.g. Zaller et al. 2011; Karasawa et al. 2012) to negative (Ayres et al. 2006). Negative growth responses can result from costs for the plant of maintaining the symbiosis that exceed the benefits particularly under conditions of high soil nutrient availability, low light intensity or weak mutual coadaptation (Johnson et al. 1997; Klironomos 2003). Lower shoot biomass may result from a mycorrhiza-induced reallocation of photosynthates from shoot to root tissue due to the higher demands of resources in roots for maintaining the mycorrhizal association. However, mycorrhizal plants in our study did not possess higher root mass either. Instead, root mass was even lower when plants were subsequently exposed

to later arriving herbivores (Figure 3d). This may indicate that higher levels of shoot consumption by later arriving herbivores further limited plant photosynthesis and thereby restricted photosynthate allocation to root tissues in mycorrhizal plants already constrained in carbon by mycorrhizal colonization. Alternatively, herbivory may have maintained plants in an induced state, conserving resources in shoots for induced defense instead of roots where mycorrhizae may directly compete for these resources.

AMF colonization influences plant IG induction and response herbivore performance

Several studies have indicated that AMF can enhance resistance against particular groups of foliar feeding herbivores, a phenomenon known as Mycorrhiza-Induced Resistance (MIR, Pozo and Azcón-Aguilar 2007; Jung et al. 2012). MIR is mainly observed for generalist chewing insect herbivores (see reviews by e.g. Koricheva et al 2009, Jung et al. 2012, Cameron et al. 2013). MIR can result from changes in primary metabolites as well as from systemic induction or jasmonic acid-dependent priming of defense metabolites (e.g., Garcia-Garrido and Ocampo 2002; Jung et al. 2012; Song et al. 2013). In *P. lanceolata*, variable effects of AMF have been observed regarding the systemic induction of its main defense metabolites, iridoid glycosides, and the ability of plants to induce these defenses in response to later arriving herbivores (Gange and West 1994; Bennett et al. 2009; Fontana et al. 2009; Schweiger et al. 2014). Our results resemble those of Bennett et al. (2009) obtained for the AMF *Scutellospora calospora* that systemically induced iridoid glycosides in the leaves of *P. lanceolata*, but suppressed a further induction of these compounds in response to herbivory. Other studies have reported either no systemic induction or even a decrease in IGs in AMF-colonized plants (Bennett et al. 2009; Fontana et al. 2009; Schweiger et al. 2014). In our study, it was mainly the concentration of catalpol that was induced by AMF, the more toxic of the two iridoid glycosides present in *P. lanceolata*, indicating that AMF can cause shifts in both the levels and in the relative proportions of iridoid glycosides in *P. lanceolata (cf.* Bennett et al. 2013). AMF caused a significant reduction in the relative growth rate (RGR) of later arriving caterpillars, which may or may not have been mediated by the induced changes in the levels of catalpol. The

reduction in RGR is consistent with the occurrence of MIR against generalist chewing foliar herbivores such as *S. exigua*. Interestingly, the AMF-induced reduction in caterpillar RGR was not accompanied by a lower relative consumption rate (RCR), and there was no significant effect of AMF on leaf area consumption. This indicates that at least in the short-term, the negative effect of AMF on *S. exigua* may have been mediated by a lower leaf quality rather than a lower feeding rate. The plant's association with AMF may therefore not directly benefit the plant in terms of reduced feeding rates of the caterpillars. However, it may potentially incur benefits in the longer run if the reduction in RGR results in lower rates of herbivore development and population growth.

AMF modulate the magnitude and timing of the defense response of plants to herbivory

Previous herbivory resulted in a reduction in leaf area consumption by later arriving herbivores when sufficient time had passed since induction took place, i.e., the effect increased over the eight-day period since the short term exposure to inducing herbivores. This indicates that herbivory results in the gradual induction of defenses that affects the consumption rate by later arriving herbivores. One of the most interesting findings of our study is that the induction of defense metabolites in response to the 24 hour period of herbivory strongly differed between mycorrhizal and non-mycorrhizal plants. When considering the subset of plants that had been exposed to previous herbivory, non-mycorrhizal plants showed a linear increase in their leaf levels of catalpol over the eight-day period, whereas mycorrhizal plants did not. One way to interpret these results is that mycorrhizae, instead of priming *P. lanceolata* plants for herbivore-induced biosynthesis of defense chemicals, actually repressed the induction of these metabolites by herbivores. Mycorrhizal suppression of the ability of plants to induce defense chemicals has been observed in *P. lanceolata* both with respect to the induction of volatile organic compounds (VOC) potentially involved in indirect defense (Fontana et al. 2009), and with respect to the induction of iridoid glycosides (Bennett et al. 2009). The extent and direction of the modulation of defense responses to

herbivory in *P. lanceolata* is AMF species dependent (Bennett et al. 2009) and further study is necessary to elucidate what governs the continuum from AMF-dependent priming to AMF-dependent repression of herbivore-induced responses in plants.

An alternative explanation for the observed lack of an herbivore-induced increase in catalpol in mycorrhizal plants in our experiments could be that in mycorrhizal plants the systemic induction of catalpol (prior to herbivory) had already resulted in the maximum amount of catalpol that could be attained in the foliage under the prevailing conditions. However, given the overall low levels of catalpol compared to levels observed in other experiments (e.g. De Deyn et al. 2009; Bennett et al. 2009), this does not seem to be a very likely explanation.

As a result of the failure of mycorrhizal plants to induce catalpol in response to herbivory, the initial difference in leaf catalpol concentrations between mycorrhizal and non-mycorrhizal plants (that did induce this compound in response to herbivory) completely disappeared after four days following herbivory. This pattern corresponds well with the observed time course of RGR and ECI of response caterpillars that initially tended to be higher on non-mycorrhizal than on mycorrhizal plants, but dropped to levels that were as low as on the mycorrhizal plants (Figure 1a, c) since four days after the herbivory. Although it is tempting to speculate that there is a causal connection between the time course of the increase in catalpol and decrease in ECI and RGR, it should be noted that the later time trend was not statistically significant. Moreover, the design of our study only allows us to speculate about the role of catalpol in mediating effects of AMF on caterpillar performance; any causal relationship is awaiting further study. Artificial diet studies have provided strong evidence that catalpol can reduce the relative growth rate of caterpillars of generalist insect herbivores including *Spodoptera* species (Bowers and Puttick 1988; Puttick and Bowers 1988). However, AM fungi are known to cause strong metabolic reprogramming of shoots; recent studies in *P. lanceolata* have shown that more than 5% of identified metabolic features changes in response to root colonization by the AM fungus

Rhizophagus irregularis (Schweiger et al. 2014). Therefore, there are probably many potential primary or secondary metabolites that could contribute to the AMF effects on herbivore growth rates. Furthermore, IGs represent a dual defense system. Upon damage, these compounds are activated by their specific beta-glucosidases (Pankoke et al. 2013). Currently it is unknown whether the activity of these beta-glucosidases is affected by AMF and/or herbivory. But if this is the case, understanding the role of IGs in mediating such interactions may be rather complex.

In summary, mycorrhizal plants had higher catalpol levels when herbivores arrived, while non-mycorrhizal plants only gradually built up this defense. Interestingly, this pattern was not explained by AMF priming of defense, but by the combination of two different AMF effects, i.e., early systemic induction and subsequent repression of the plant's ability to exhibit an herbivore-induced response. Its causal role in modulating the herbivore response awaits further study.

Conclusions

In our study AMF caused a reduction in plant biomass, but also resulted in a systemic increase in the concentration of defense metabolites in the shoots of *P. lanceolata.* This could have contributed to the negative impact of AMF on the performance of later-arriving shoot herbivores. Non-mycorrhizal plants only reached these levels of defense metabolites eight days after induction by herbivores, while levels of defense compounds in mycorrhizal plants were not affected by herbivory. Our study thus reveals that AMF can modulate the time course of effects of previous herbivory on plant responses to, and performance of, later arriving herbivores, which may in turn determine plant performance and fitness in the longer run. This highlights the importance of including temporal aspects in future research on interactive aboveground-belowground impacts of herbivory and AMF on expression and effects of induced plant defenses.

Acknowledgements

Chapter 4

Plant responses to variable timing of aboveground clipping and belowground herbivory

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To be submitted

Abstract

Plants can use tolerance and induced defense to mitigate the effects of herbivores. The direction and magnitude of both these plant responses to herbivory can vary with plant ontogeny. We exposed the perennial grass species *Holcus lanatus* to foliar clipping and root-feeding nematodes at three growth stages (young, intermediate and old plants), and examined plant tolerance and defense responses. We measured changes in the level of primary (C, N) and secondary (total phenolics) compounds in plant foliage and roots three weeks after defoliation and nematode addition ("short term"). Addition of nematodes and defoliation increased foliar N concentrations, but only when applied to intermediate and old aged plants. Nematode addition and defoliation further caused a reduction in the foliar concentration of total phenolics, regardless of the growth stage of the plant at which nematodes had been added and defoliation was initiated. Primary and secondary compounds were also measured at a fixed plant age, 12 weeks after the initiation of experiment ("long term"). The only significant effect was observed in the treatment where nematodes had been added when plants were young. These plants had reduced root N concentrations. Relative shoot regrowth rates tended to be increased immediately after defoliation, but they decreased with time after clipping and this was independent of plant age at clipping. Similarly, relative root growth rates increased shortly after adding nematodes and defoliation, and then declined again, but this was only observed when the treatments were applied to young plants. In contrast, intermediate-aged plants showed longer term enhancement of relative root growth rates following the addition of nematodes and defoliation compared to control plants. We conclude that defoliation and nematode addition transiently increased plant foliar quality (higher %N, lower concentration of total phenolics), independent of plant age. In contrast, in the short term root quality was not influenced by defoliation at any plant age, but the magnitude of the relative root growth rate following defoliation highly depended on plant ontogenetic stage.

Introduction

Plants can be exposed to various above- and belowground herbivores during their life. To reduce loss of fitness, plants have evolved strategies to mitigate the negative effects of these herbivores, for example by induced defense (Karban and Baldwin 1997) or tolerance (Caldwell et al. 1981). Induced defense enables plants to deter contemporary or future herbivores by producing higher levels of secondary compounds, while plants can also reduce consumption by herbivores by lowering nutrient content of plant tissues (Agrawal 1998). Other mechanisms to deal with herbivores are tolerance of herbivore damage through mechanisms that reduce the negative fitness effects of damage incurred by the herbivores (McNaughton 1983). Both defense strategies are widely identified and incorporated in ecological and evolutionary theories on plant-herbivore interactions (Tollrian and Harvell 1999; Strauss and Agrawal 1999).

The induction of plant defenses can vary with plant age as the priority to defend different plant organs changes during plant growth (Boege and Marquis 2005; del-Val and Crawley 2005; Barton 2007; Akiyama and Ågren 2012). Many studies have shown that the inducibility of metabolic compounds varies between ontogenetic stages of the plant (McArthur et al. 2010; Quintero and Bowers 2012). For example, Quintero and Bowers (2011) investigated the chemical responses of *Plantago* spp. to a specialist foliar feeding insect herbivore and found that only in juvenile and not in mature plants, plant defense was induced in response to herbivory. In contrast, other studies have shown that older plants defend themselves against herbivores by accumulation of higher concentrations of plant defense compounds over a longer growth period (Elger et al. 2009; Goodger et al. 2004; Boege 2005). These studies mainly examined how the concentration of defense compounds changes following a single damage over plant age and often show that plant age at herbivory is important for induction of plant defense (e.g. Quintero and Bowers 2011). However, only few studies have examined plant defense responses to herbivore damage throughout the development of the plant.

A plant's ability to tolerate herbivore damage can also vary with plant age (Massad 2013) due to developmental changes in plant architecture, storage capacity, and resource allocation (Boege 2005). In general, young plants possess fewer stored reserves and lower capacity of resource acquisition, so that they are less capable of compensating herbivore damage than old plants (Bryant et al. 1992). Other studies have shown that plant growth was more strongly suppressed by herbivory in older plants, because that coincides with the plant's allocation of resources to reproduction (Nykänen and Koricheva 2004). Interestingly, intermediate-aged plants can be more vulnerable than both young and old plants due to the lack of seed-stored reserves in comparison to young plants and the lack of photosynthetic area in comparison to older plants (del-Val and Crawly 2005). Plants usually differ in many intrinsic traits such as the ability to store and translocate resources (Trumble et al. 1993) or ability to increase photosynthesis (Nykänene and Koricheva 2004) and growth rates (Tiffin and Inouye 2000) over age. These traits can determine a plant's tolerance to herbivory and the ontogenetic pattern of these traits may reflect adaptive plasticity under variable herbivore pressures (Pankoke et al. 2013).

The majority of studies on plant responses to herbivory have focused on aboveground effects, whereas there have been relatively few studies on consequences of belowground herbivory (van Dam 2009). Root-feeding nematodes are major root herbivores of many plant species (Perry and Moens 2006), and they are frequently studied in interaction with their host plants (Mateille 1994; van Dam et al. 2003; Soriano et al. 2004; Zinov'eva et al. 2004). Many studies have shown that the presence of root-feeding nematodes can greatly alter the metabolite profile of host plants, in particular the concentration and composition of plant defense compounds (van Dam et al. 2005; Kaplan et al. 2008b; Hol et al. 2010; de la Peña and Bonte 2014), or amino acids (Bezemer et al. 2005). Other studies have shown that in some cases nematode feeding does not result in changes in primary or secondary compounds in the host plant per se (Kutyniok and Müller 2012), but that they have an effect on concentrations of these compounds in the presence of other organisms, such as earthworms (e.g. Lohmann et al. 2009).

Many pot studies reported minor effects of root-feeding nematodes on plant growth (Seastedt et al. 1987; Verschoor et al. 2002). Exposure to root-feeding nematodes can reduce (Stanton et al. 1981; Ingham and Detling 1990; Brinkman et al. 2008) but also enhance plant biomass (Stanton 1983) depending on the studied plant and nematode species (Verschoor et al. 2002; Brinkman et al. 2015). The responses of primary or secondary plant compounds to nematode herbivory also vary with nematode species (Vaast et al. 1998), plant traits (Verschoor et al. 2002) and soil conditions (De Ruijter and Haverkort 1999). The variation among these studies suggests specific interplays between plant and root-feeding nematode assemblages.

In this study, we examined how clipping of foliar material and addition of root-feeding nematodes influenced plant growth and chemistry. We used clipping as a surrogate for herbivory, but we acknowledge that clipping differs from herbivory as it is not selective and lacks effects of elicitors and effectors from herbivore oral secretions. We exposed the perennial clonal grass *Holcus lanatus* to these herbivory treatments at young, intermediate and old age. The aim was to examine plant responses to damage by above- and/or belowground herbivory in relation to plant ontogeny. We chose *Holcus lanatus* because it has been described as a plant species that has evolved tolerance and defense to cope with biotic stresses (Tiffin and Inouye 2000). We hypothesized that 1) clipping and nematode addition will induce a higher level of general plant defense (phenolics), and a lower nutritional value (N concentration), but that the defense level will depend on plant age. (2) Plant growth following defoliation and nematode herbivory will increase with plant age, as old plants have more stored resources available to biomass regrowth; and (3) Plant growth rate will decrease with time after defoliation and exposure to nematodes.

Materials and Methods

Soil, plants and nematode inocula

We collected soil from a restored grassland on former arable land (De Mossel, Ede, The Netherlands, 52.04 °N 5.44 °E) at 5-20 cm below the soil surface. The soil was a sandy loam with 4.5% organic matter. In the laboratory, the soil was sieved through a 5 mm mesh, homogenized and sterilized by gamma irradiation (>25 KGray). Our focal grass species *Holcus lanatus* is a perennial grass species that commonly occurs in most European grasslands on various soil types (Beddows 1961), including the site where soil was collected. It is used as a host by a variety of root-feeding nematode species (Verschoor et al. 2001, De Deyn et al. 2004). Seeds of *Holcus lanatus* were purchased from a commercial wild-seed supplier (Cruydt-hoeck, Nijeberkoop, the Netherlands). The seeds were surface sterilized with sodium hypochlorite (1%) for 1 min and rinsed 4 times with demineralized water, sown on moist glass beads and then placed in an incubator (16/8 h light/dark, 25/20 °C day/night temperature) until germination.

Nematodes were collected from a field that has been cultivated for agriculture since 1955 (Vredepeel, the Netherlands) with annual mean temperature of 10.2 °C and precipitation of 766 mm (Korthals 2014). The soil contained 1.1% clay, 3.7% silt and 94.9% fine sand. The nematode inoculum was dominated by root-feeding nematodes and mainly contained the species *Pratylenchus penetrans* and *Tylenchorhynchus dubius* (98.4% of the root-feeding nematodes). As the study was designed to repeatedly inoculate nematodes (see below) these nematodes had been collected at different times from February to April in 2014. Each time, soil samples were collected from the same location within the agricultural field. However, because the nematodes were collected at different times in the season, the composition of the nematode communities that we used as inoculum in the experiment varied between inoculation dates (Table S4.1). By adjusting the inoculation volume, densities of plant feeding nematodes that we inoculated per plant were roughly the same throughout the experiment.

Experimental setup

We set up the experiment with a total of 320 pots filled with 800 g fresh soil each, and one seedling of *H. lanatus* was planted into each pot. All pots were positioned randomly in a greenhouse (16/8 h light/dark, 21/18±2 °C day/night). The pots were then assigned to one of the following treatments: (i) addition of nematodes (belowground herbivory, BG) (ii) addition of nematodes and defoliation (AG+BG), and (iii) control. The treatments were applied at three different plant growth stages (young, intermediate, old).

Young plants: At week 0, three weeks after transplanting, 110 randomly chosen pots were inoculated with 4 ml nematode suspension. The suspension contained on average 100 (SE = 6.8) individuals of *P. penetrans* and *T. dubius* and was inoculated into two 1-cm-deep small holes made in the soil. The holes were immediately covered using the surface layer of the soil in the pot. In week 1, ten randomly selected plants were harvested and used to examine nematode survival (Figure S4.1). Meanwhile, 50 of the remaining plants were defoliated by clipping at 4 cm above the soil surface (AG+BG-Y(oung) treatment). The other 50 remaining plants were the nematode treatment (BG-Y treatment).

In week 3, when plants were six weeks old and named intermediate-aged, 90 randomly chosen pots were inoculated with 5 ml nematode suspension that contained 100 (SE = 15.7) per 5 ml individuals of *P. penetrans* and *T. dubius* as described above. One week later (experimental week 4), ten of the plants were harvested to check nematode survival (Figure S4.1). We defoliated 40 plants (AG+BG-I(ntermediate) treatment) as previously described while the other 40 plants only had nematodes added (BG-I treatment).

In week 6, when plants were nine weeks and named old, 70 randomly chosen plants were inoculated with 5 ml nematode suspension containing 100 (SE = 5.7) per 5 ml individuals of *P. penetrans* and *T. dubius*. One week later (experimental week 7), ten plants were harvested to check nematode survival
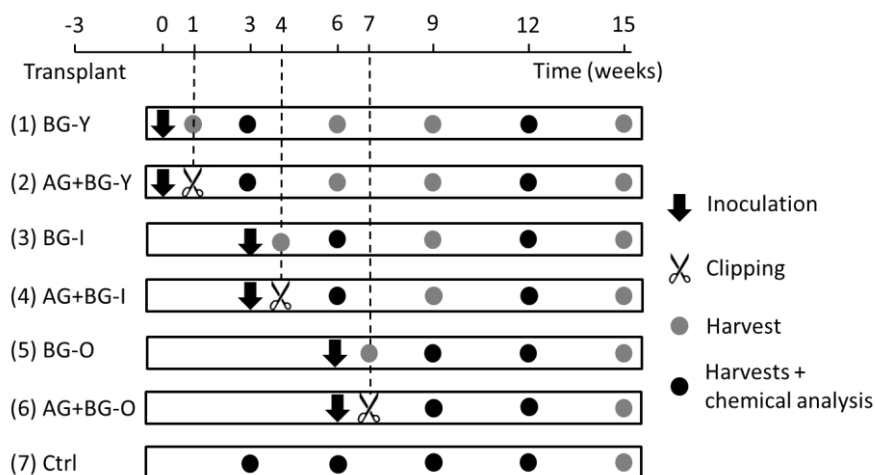
Figure 4.1 Scheme of harvest and treatments of the experiment. Belowground (BG) treatments: plants were inoculated with the root-feeding nematode species *P. penetrans* and *T. dubius* at weeks 0 (Young; capital used for abbreviating), 3 (Intermediate), and 6 (Old). Belowground and aboveground (BG+AG) treatments: plants were defoliated 1 week after inoculation with root-feeding nematodes. The control (Ctrl) treatment refers to plants that were neither inoculated with nematodes nor defoliated. Subsets of plants were harvested every 3 weeks after nematode inoculation until the last harvest at week 15. Arrows indicate the nematode inoculation events. Scissors indicate the defoliation events and grey circles indicate the harvests within treatments. The filled circles indicate treatments selected for subsequent chemical analysis. One week after inoculation 10 plants were harvested to check nematode survival (dashed lines).

(Figure S4.1). We defoliated 30 plants (AG+BG-O(ld) treatment) while the other 30 plants were not defoliated (BG-O treatment).

Control plants (Ctrl treatment) were inoculated with tap water at experimental weeks 0, 3, and 6, using the same method as described for nematode inoculation. For all treatment and plant age combinations, ten plants were harvested every three weeks following nematode inoculation until week 15 ("sequential harvests") (Figure 4.1).

Plants were watered three times per week. Once a week, the soil moisture content was reset to 12.3 % (w/w) by weighing using demineralized water. Nutrients were added once a week using Hoagland solution (Hewitt 1966). The nutrient strength and dosage were gradually increased over time to meet

growth demands of *H. lanatus* (Van der Putten et al. 1988) according to ontogenetic measurements of nitrogen concentration for *H. lanatus* (T.M. Bezemer, unpublished data). A quarter-strength Hoagland was added in weeks 1-4 (from 12.5 ml to 50 ml per week in steps of 12.5 ml), half-strength solution was added in weeks 5-9 (from 60 ml to 100 ml per week in steps of 10 ml), and full-strength solution was added in weeks 10-14 (from 60 to 100 ml per week in steps of 10 ml). The experiment was carried out in a greenhouse with natural daylight supplemented by 400-W metal halide lamps (225 µmol m^{-2} s^{-1} photosynthetically active radiation) and every week, the pots were rotated within the greenhouse in order to minimize effects of local site differences.

Plant harvest and chemical analysis

Biomass At each harvest, the shoots were separated from the roots using scissors and the soil was carefully washed off the roots. Shoot and root biomass were oven-dried at 40 °C for a minimum of 5 days before weighing (Figure S4.2). Seven randomly selected plants from each of the treatments harvested at three weeks after inoculation, and from all treatments harvested at week 12 ("fixed harvest") were used for further chemical analysis (see Figure 4.1).

C, N concentration The dried shoots and roots of the selected plants were ground and 1 mg was weighed into tin capsules. Carbon (C) and nitrogen (N) concentrations were measured using a CN analyzer (Flash EA 1112, Interscience, Breda, The Netherlands).

Total phenolics Total phenolic concentration was determined using the Folin-Ciocalteu method (Medina-Remón et al. 2009). Twenty five mg of ground dry plant material was extracted with 5 ml 50% methanol for 2 hrs in a water bath at 90 °C. The samples were centrifuged for 10 min at 5000 rpm and the supernatant was analyzed. Two-hundred µl supernatant was mixed with 200 µl Folin-Denis reagents and 1.0 ml Na_2CO_3 solution and centrifuged at 12000

rpm for 5 min. The supernatant was measured at a wavelength of 750 nm. Tannic acid was used as a standard.

Data analysis

To examine the effects of defoliation and nematode addition at different plant ages, we used data from the harvests three weeks after nematode inoculation ("sequential harvests", i.e. weeks 3, 6 and 9 for plants inoculated at young, intermediate, and old ages, respectively). We used a two-way ANOVA to analyze plant biomass, C, N and phenolic concentration in plant shoots and roots. There were two treatments: 'herbivory' (Ctrl, BG and AG+BG) and plant 'age' (young, intermediate and old), which were used as fixed factors. Tukey HSD *post hoc* tests were used for multiple comparisons when treatment effects were significant. Further, we analyzed biomass and chemistry data of plants of all treatments harvested at week 12 ("fixed harvest") using a one-way ANOVA. Subsequently, we used a Dunnett *post hoc* test to compare the control treatment with each of the BG or AG+BG treatments at each of the three plant ages. In addition, we used a two-way ANOVA to test whether defoliation (BG vs AG+BG) and plant age (young, intermediate, old) affected plant biomass and chemistry, while excluding the control treatment.

We used linear regression to test the relationship between biomass and phenolic concentration in plant shoots and roots at week 12 (Figure S4.3).

We also calculated temporal changes in plant shoot and root biomass following the start of the different treatments. Proportional biomass change (PBC) was calculated as PBC = $(B_x-B_{x-3})/B_{x-3}$, where B_x is the biomass (shoot or root) of an individual plant at weeks x = 6, 9, 12 and week 15. We used these relative root and shoot growth rates as proxies for the ability to regrow following defoliation as an aspect of tolerance. As we destructively harvested plants and thus could not match individual plants between two harvests, we randomly paired the ten plants from the same treatment from two consecutive harvests to calculate PBC values. We analyzed the data using a two-way ANOVA with 'herbivory' treatment (AG+BG, BG and Ctrl) and 'time' (weeks

3-6, 6-9, 9-12 or 12-15) as fixed factors. We repeated this procedure 1000 times, which yielded 1000 ANOVA results. The number of significant occurrences of each main factor and interaction (at $p<0.05$ level) out of 1000 replicates were summed and the proportion of non-significant occurrences was calculated (*Pr* = number of non-significant occurrences out of 1000). The factors herbivory and time with *Pr*<0.05 were considered significant. To determine whether two treatments differed significantly, a *contrast test* was used in which we compared the BG treatment with Ctrl and with AG+BG, whereas the AG+BG treatment was also compared with the Ctrl. The *contrast test* was also replicated 1000 times and the *Pr* values were calculated as previously described for each contrast.

Root biomass from the sequential harvests was log-transformed to meet the ANOVA assumptions. All analyses were performed using the R statistical package, version 3.1.3 (R Core Team 2014).

Results

Plant biomass

Three weeks after inoculation (i.e. weeks 6, 9 and 12 for plants inoculated at early, intermediate and old age, respectively), AG+BG herbivory had substantially reduced plant biomass compared to Ctrl, whereas there was no significant effect of only nematode addition and these effects were independent of plant age at defoliation (Table 4.1, Figure 4.2a). For all ages at which we applied the herbivory treatments, the reduction of shoot biomass by AG+BG herbivory was also significant at week 12 (Table 4.1, Figure 4.2b). Root biomass was reduced in treatments with nematode addition (BG) but only when the nematodes were inoculated at intermediate plant age (Table 4.1, Figure 4.2c). There were no significant nematode addition effects at week 12 regardless of plant age at nematode addition (Contrasts: Ctrl vs. BG-Y, Ctrl vs. BG-I, Ctrl vs. BG-O; *Dunnett* tests: all P > 0.10, Fig 4.2d). Root biomass was consistently reduced by AG+BG herbivory both when measured three weeks after inoculation and for all treatments measured at week 12 (Comparison:

Ctrl vs. AG+BG) and the AG+BG effect was not influenced by plant age at herbivory (Table. 4.1, Figure 4.2c, 4.2d).

Table 4.1 ANOVA results for effects on plant biomass of herbivory and plant age at time of herbivory (Age). Left columns (sequential harvests) are analyses of plant biomass three weeks after nematode inoculation and right columns (Harvest at 12 weeks) are analyses based on all plants harvested at the same time (12 weeks) of growth.

| | | Sequential harvests | | | | | Harvest at week 12 | | | |
| | | Shoot | | Root | | | Shoot | | Root | |
	df	F	p	F	p	Df	F	p	F	p
Herbivory[b]	2	236.93	**<0.001**[a]	68.90	**<0.001**	1	82.30	**<0.001**	9.48	**0.003**
Age[c]	2	713.37	**<0.001**	289.93	**<0.001**	2	7.04	**0.002**	0.07	0.933
Herbivory×Age	4	34.31	**<0.001**	4.95	**0.001**	2	5.28	**0.008**	0.41	0.669
Error	81					54				

[a] Bold p values indicate significant effects at P < 0.05.

[b] Herbivory indicates treatments assigned to "Ctrl", "BG" and "AG+BG".

[c] Age indicates treatments with nematode inoculation at weeks 0, 3 and 6.
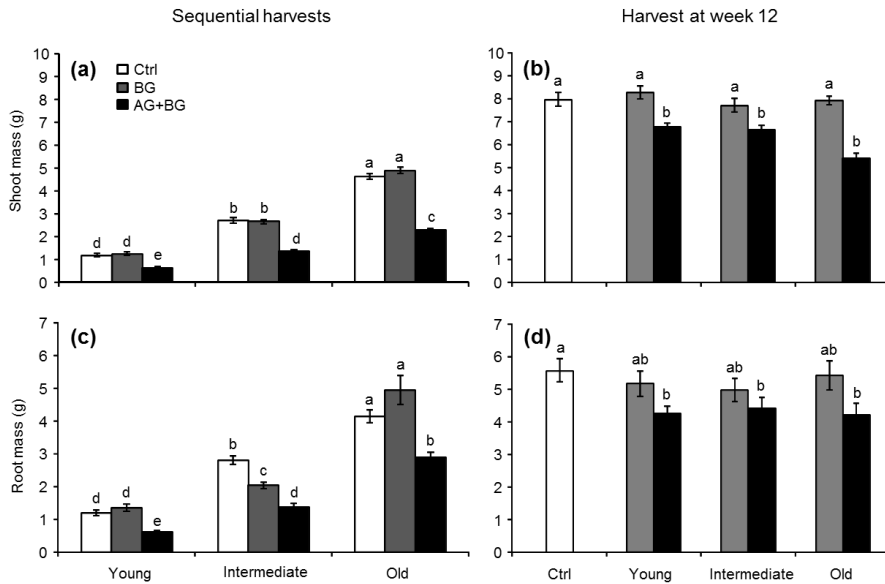

Figure 4.2 Shoot (**a** and **b**) and root (**c** and **d**) biomass of plants exposed to nematodes (BG, grey bars) and both nematodes and defoliation (AG+BG, dark bars) or plants neither exposed to nematodes nor defoliation (Ctrl) at different plant ages (Young, Intermediate, Old). Biomass of plants were sequentially harvested 3 weeks after each inoculation (panels **a** and **c**) and at week 12 (panels **b** and **d**). Within panels, bars with identical letters are not significantly different (*P <0.05)* based on a Tukey HSD test.

Table 4.2 ANOVA results for effects on plant chemistry (%N, %C, and total phenolics) of herbivory and plant age at the time of herbivory (Age). Upper rows are analyses of plant chemistry three weeks after herbivory and lower rows are analyses of plant chemistry at the same time (12 weeks) of growth.

			% N (DW)				% C (DW)				Total phenolics (mg/g DW)			
			Shoot		Root		Shoot		Root		Shoot		Root	
		df	F	p	F	p	F	p	F	p	F	p	F	p
Sequential harvests	Herbivory[b]	2	43.21	<0.001[a]	1.10	0.341	0.31	0.732	4.49	**0.016**	7.93	**0.001**	1.64	0.203
	Age[d]	2	5.27	**0.008**	8.96	**<0.001**	3.36	**0.042**	2.22	0.118	1.37	0.263	0.27	0.763
	Interaction	4	8.38	**<0.001**	3.01	**0.026**	9.93	**<0.001**	3.35	**0.016**	0.44	0.779	2.87	**0.032**
	Error	54												
Harvest at week 12	Herbivory[c]	1	1.66	0.206	0.02	0.905	0.02	0.878	0.01	0.956	0.90	0.349	0.01	0.942
	Age[d]	2	1.94	0.159	2.39	0.106	8.57	**0.001**	1.86	0.171	2.89	0.069	0.42	0.663
	Interaction	2	0.56	0.577	0.14	0.866	0.54	0.587	0.19	0.188	0.69	0.510	4.14	**0.024**
	Error	36												

[a] Bold p values indicate significant effects at $P < 0.05$.

[b] In Sequential harvests, herbivory indicates treatments assigned to "Ctrl", "BG" and "AG+BG".

[c] In Harvest at week 12, herbivory indicates treatments assigned to "BG" and "AG+BG".

[d] Age indicates treatments with nematode inoculation at weeks 0, 3, and 6.

Primary and secondary metabolites

Leaf N concentration was increased by AG+BG herbivory, but only in plants exposed at intermediate and old age (Table 4.2, Figure 4.3a). At week 12, neither BG herbivory nor AG+BG herbivory resulted in a significant change in leaf % N; all contrasts of Ctrl vs. BG-Y, Ctrl vs. BG-I, Ctrl vs. BG-O, and BG vs. AG+BG, had a P > 0.10 using Dunnett tests (Table 4.2, Figure 4.3b). Root % N was not influenced by nematode additions three weeks after inoculation (Comparison: BG vs. Ctrl, Table 4.2, Figure 4.3c). However, nematode addition reduced root % N at week 12, when plants had been inoculated at young age (Contrast: Ctrl vs. BG-Y, *Dunnett* test: P = 0.004, Figure 4.3d). Root % C was enhanced by AG+BG herbivory, but only in plants inoculated and defoliated at old age (Table 4.2, Figure 4.4c). Shoot % C was not changed by BG or AG+BG herbivory across plant ages (Figure 4.4a). There were no herbivory or plant age effects on % C in roots or shoots when measured at week 12 (Table 4.2, Figure 4.4b and 4.4d). Three weeks after inoculation, total phenolic concentrations in shoots were reduced by AG+BG herbivory, irrespective of plant age (Table 4.2, Figure 4.5a). However, the concentration of total phenolics in roots was not significantly affected by herbivory (Figure 4.5c). At week 12, there were no significant effects of herbivory on concentration of total phenolics (Table 4.2, Figure 4.5b and 4.5d).

Relative plant shoot and root biomass changes over time

Young plants showed higher relative increases in shoot biomass when exposed to AG+BG herbivory compared to the BG herbivory, but the difference in increase did not persist in time, resulting in a significant Herbivory×Time interaction (Contrast AG+BG vs. BG, *Pr* < 0.05, Figure 4.6a). A similar effect on proportional shoot biomass change was observed in intermediate and old aged plants exposed to AG+BG herbivory (Contrast AG+BG vs. BG, both *Pr* < 0.05, Figure 4.6a, 4.6c, 4.6e). Nematode addition alone did not affect relative shoot biomass change (Contrasts: BG vs. Ctrl, all
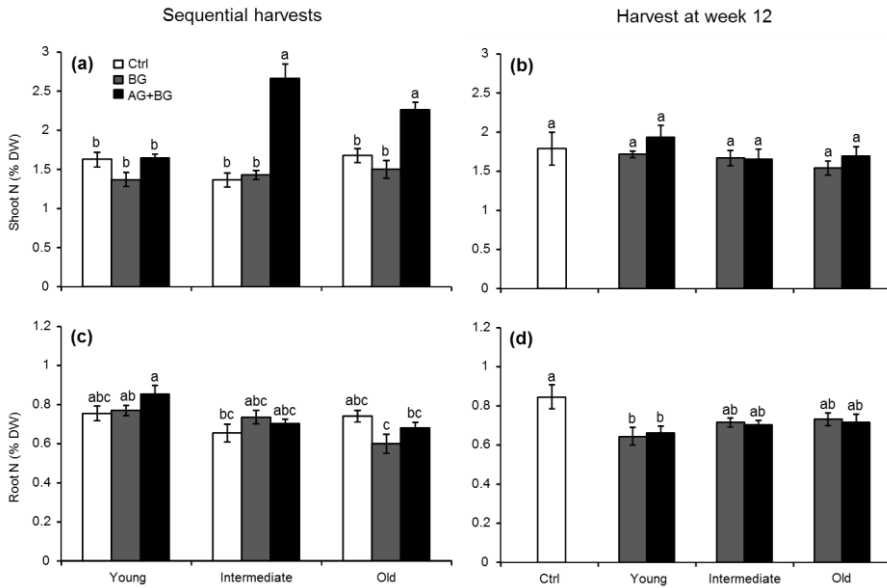
Figure 4.3 Percentage N in shoots (**a** and **b**) and roots (**c** and **d**) of plants exposed to nematodes (BG, grey bars) and both nematodes and defoliations (AG+BG, dark bars) or plants neither exposed to nematodes nor defoliation (Ctrl, open bars) at different plant ages (Young, Intermediate, Old). Both % N in plants sequentially harvested 3 weeks after inoculation (panels **a** and **c**) and at week 12 (panels **b** and **d**) are shown. Within panels, bars with identical letters are not significantly different (*P =0.05*) based on a Tukey HSD test.

Pr > 0.10, Figure 4.6a, 4.6c, 4.6e). AG+BG herbivory also caused a significant relative increase in root biomass at young plant age, but the effect disappeared over time (Herbivory×Time interaction: *Pr < 0.05*, contrast AG+BG vs. BG, Figure 4.6b). At intermediate plant age herbivory constantly increased the proportion of root biomass change over time (AG+BG vs. Ctrl: *Pr* < 0.05, Figure 4.6d). However, in old plants the proportion was not influenced by herbivory (AG+BG vs. Ctrl: *Pr* > 0.10, Figure 4.6e). Nematode addition also did not affect proportion of root biomass change and this absence of effect was independent of plant age at defoliation (BG vs. Ctrl: all *Pr* > 0.10, Figure 4.6b, 4.6d, 4.6f).
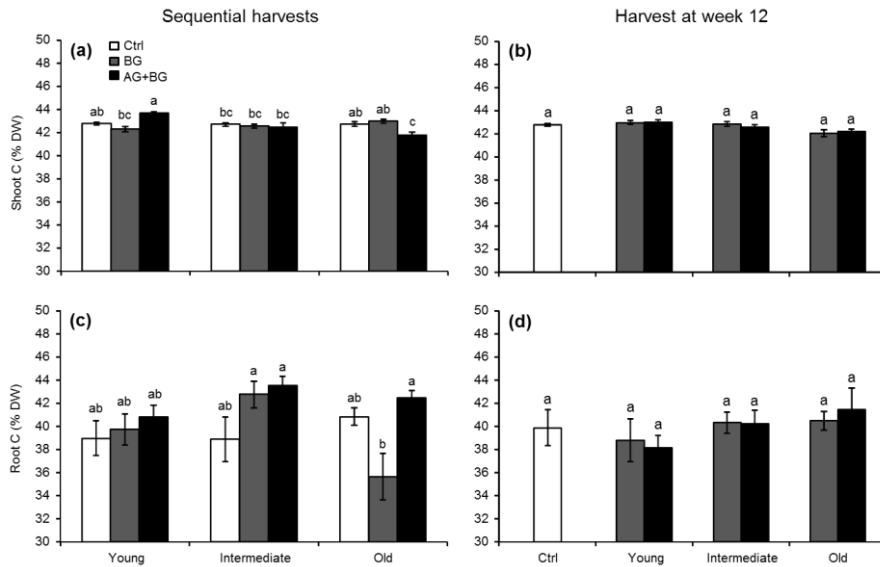
Figure 4.4 Percentage of C in shoots (**a** and **b**) and roots (**c** and **d**) of plants exposed to nematodes (BG, grey bars) and both nematodes and defoliations (AG+BG, dark bars) or plants neither exposed to nematodes nor to defoliation (Ctrl, open bars) at different plant ages (Young, Intermediate, Old). Both % C in plants sequentially harvested 3 weeks after inoculation (panels **a** and **c**) and at week 12 (panels **b** and **d**) are shown. Within panels, bars with identical letters are not significantly different (*P<0.05*) based on a Tukey HSD test.

Discussion

We exposed the grass species *Holcus lanatus* to defoliation and root-feeding nematodes and examined how plant regrowth, nutritional, and defensive responses would be affected by plant age. Even though defoliation does not really mimic herbivory, we found that defoliation and nematode addition transiently increased leaf nitrogen concentrations of regrown foliage and reduced the concentration of phenolics in these leaves, which might result in altered plant susceptibility to generalist herbivores. This increase was not affected by plant age of defoliation. Inoculation of root-feeding nematodes did not have a significant effect on plant chemistry, except a decrease in the root nitrogen concentration in the longer term. Compensatory regrowth of shoot biomass following defoliation took place only shortly after defoliation and this was independent of plant age at defoliation. Root growth after defoliation

was plant-age specific: defoliated intermediate-aged plants had a longer time of enhancement in growth rates than young plants while old plants were not enhanced in root growth rates after defoliation. Both shoot and root growth after defoliation tended to decrease with progressing time since defoliation. Our results suggest that grasses can show a higher response in tolerance than in defense following herbivory (Stanton 1988), and the root growth can be more plastic than shoot growth in response to defoliation over development.
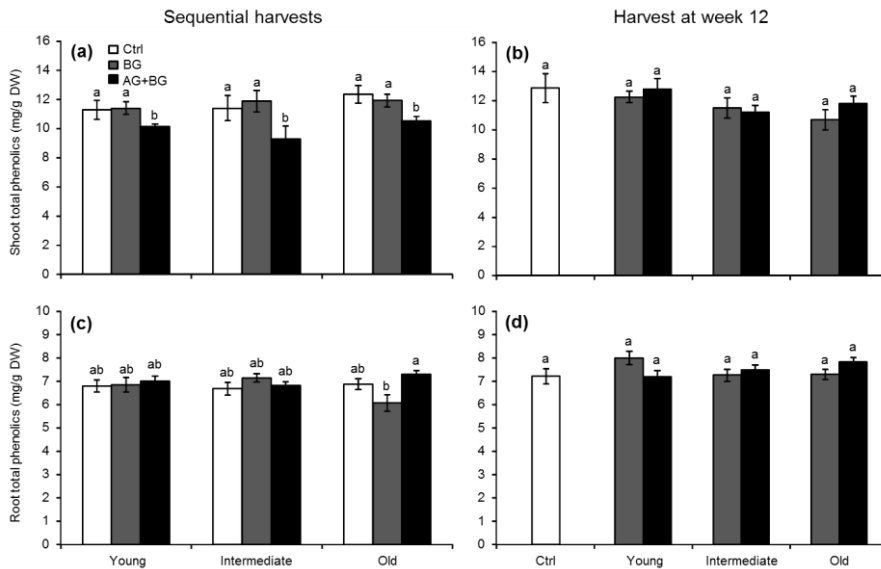

Figure 4.5 Shoot (**a** and **b**) and root (**c** and **d**) concentration of total phenolics in plants exposed to nematodes (BG, grey bars) and both nematodes and defoliations (AG+BG, dark bars) or in plants neither exposed to nematodes nor to defoliation (Ctrl, open bars) at different plant ages (Young, Intermediate, Old). Both total phenolics concentration in plants sequentially harvested 3 weeks after inoculation (panels **a** and **c**) and at week 12 (panels **b** and **d**) are shown. Within panels, bars with identical letters are not significantly different ($P < 0.05$) based on a Tukey HSD test.

Plant age effects on induced defense following clipping and nematode exposure

Induced defense theory predicts that plant defense can be induced by herbivory and that this results in higher levels of plant defense compounds (Karban and Baldwin 1997). Opposite to the prediction, we observed transiently lower concentrations of total phenolics in defoliated plants. Earlier

studies also reported that foliar herbivory could result in a local decrease in the concentration of secondary compounds, particularly when available resources for metabolite synthesis were limited and simultaneously competed for by other functions such as growth and storage (Thomson et al. 2003). As herbivory in our study was restricted to the vegetative phase of a grass species and plants were well fertilized and watered throughout experiment, the reduced concentration of foliar phenolics should not be the consequence of resource constraints or poor plant functioning. Although grass species are widely accepted to be tolerant to aboveground herbivory they can also produce a variety of secondary chemicals as defense (Redak 1987). Hence plant compensatory growth and defense activity may compete for plant stored resources when photosynthesis is limited at defoliation (Boege 2005). We expected an influence of plant age at defoliation on foliar levels of plant defense metabolites, but this effect was not observed for phenolics in the present study (van Dam and Baldwin 2001). Possibly, this lack of effect is related to the fact that the level of total phenolics of *H. lanatus* appeared to be independent of plant age: the constitutive level of total phenolics in control plants did not change with plant age in the current study (Figure 4.5a). Alternatively, the composition rather than concentration of phenolics may have been altered across plant ages at defoliation, which can also affect plant defenses against herbivores (Donaldson et al. 2006).

Nematode addition can also significantly change the concentration of secondary plant compounds in plants (e.g. van Dam et al. 2005). Nematodes can significantly reduce plant root biomass, resulting in a decrease of nutrient uptake. A decrease of nutrient availability to plants is usually accompanied by C accumulation that can be available for synthesis of C-intensive defense compounds (Larson 1986; Cronin and Lodge 2003), although this can be counteracted by an increased sink strength of local feeding structures of sedentary endoparasitic nematodes (Cabello et al. 2014). In the current study, nematode addition reduced the root biomass in *H. lanatus* at intermediate age, but neither C concentration nor concentration of total phenolics were altered. Possibly, *H. lanatus* is not strongly responsive to these nematodes or this plant species does not deploy defense as a priority after exposure to nematode

feeding (Barton and Koricheva 2010). This result indicates that root-feeding nematodes may influence plant performance not by altering plant defense status, but because *H. lanatus* tolerates a fair amount of nematodes feeding on its roots. Similar effects have been shown for other grass-nematode combinations (Brinkman et al. 2015).

Plant nitrogen responses to clipping and nematode addition during ontogeny

Plant regrowth after defoliation primarily relies on mobilization of available photosynthates. Photoassimilates are preferentially allocated to active shoots that cause a sink activity (Briske and Richards 1995). The assimilate allocation to shoots can occur at the expense of root growth (Ryle and Powell 1975). A decrease of resource allocation to roots following defoliation can lead to root mortality and a reduction in root growth and nitrogen uptake (Kosola et al. 2001; Boege 2005; Deslauriers et al. 2015). Consequently the N content could be reduced in defoliated plants. In contrast, we found that foliar N concentration in regrown plant foliage was enhanced by defoliation and nematodes two weeks after defoliation. Enhanced contents only occurred in plants at intermediate and old age. The higher N content in plant shoots can be remobilized from remaining shoot tissues or allocated from the root system (Ourry et al. 1990). Following defoliation intermediate and old plants possess higher amounts of remaining shoot tissues so they are also likely to have more previously absorbed N than young plants. On the other hand, the enhanced N concentration in plant foliage may also result from a redirection of N from roots to shoots after defoliation (Millard and Robinson 1990; Ourry et al. 1990). Young plants have smaller N pools in roots and lower ability in N uptake after defoliation, which constrains the amount of root N that can be mobilized to shoots. This may also partly explain the higher N concentration observed in intermediate-aged and old plants. Opposite to an increase of N concentration in plants following defoliation above ground, we found a decrease of root N concentration in plants with nematode addition at 12 weeks. However, the decrease only occurred in young plants probably because young plants had suffered the longest feeding period and severest suppression on N absorption and assimilation.
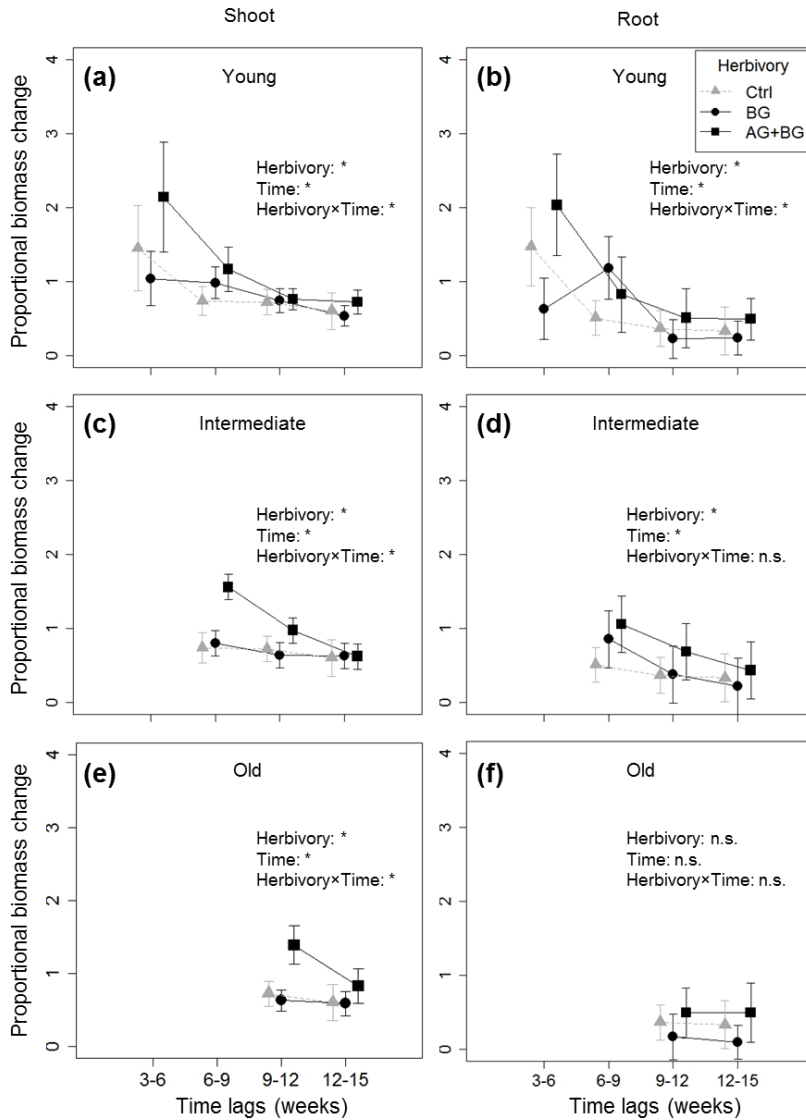
Figure 4.6 Proportional biomass change (PBC, PBC = $(B_x-B_{x-3})/B_{x-3}$, B_x indicates biomass at week x) in shoots (panels **a**, **c**, **e**) and roots (panels **b**, **d**, **f**) over time ("Time", time lags after each defoliation) when plants were exposed to nematodes (BG) or both nematodes and defoliation (AG+BG) or neither exposed to nematodes nor defoliation (Ctrl) at young (**a** and **b**), intermediate (**c** and **d**) and old ages (**e** and **f**). The asterisks indicate statistical significances at *Pr < 0.05* based on a bootstrap analysis and n.s. means non-significant. The graphs were made based on the mean and error bars within 95% confidence intervals that were calculated from 1000 replicate analyses (see data analysis).

Plant biomass in response to clipping and nematode addition during ontogeny

In accordance with many other studies, final plant biomass was significantly reduced by defoliation (McNaughton 1983; Oesterheld 1992; Painter and Belsky 1993). The removed biomass can be compensated by the plant over time after defoliation (McNaughton 1983) and the extent of compensation depends on many factors including timing of defoliation (Boege 2005) and the amount of time available for recovery (Oesterheld and McNaughton 1991). In a period of two weeks after defoliation, the regrowth of shoot biomass following AG+BG herbivory was independent of plant age at herbivory (Figure 4.2). In our study, at 12 weeks after experimental initiation, plants defoliated at variable ages differed in the time that was available for recovery. Young plants had the longest time for regrowth, thus they were predicted to show a higher extent of compensation in comparison to plants defoliated at intermediate and old ages (Oesterheld and McNaughton 1991). In contrast to this prediction, all plants compensated shoot biomass to a similar extent independent of age at defoliation (Figure 4.2), in accordance with the view that compensatory regrowth rates will depend on plant age and that older plants can more rapidly compensate for removed biomass (Hilbert et al. 1981). In line with other studies using different plant species (Brandt and Lamb 1994; Warner and Cushman 2002; Boege et al. 2007), these results collectively reflect increased tolerance as plant age (Boege et al. 2007). Nematode addition significantly reduced root biomass but only of plants at intermediate age three weeks after inoculation. This may be because young plants usually have a higher growth rate (Walters et al. 1993) and old plants can better compensate loss to herbivory (Elger et al. 2009), thereby mitigating or even counteracting the negative effects of root-feeding nematodes on root biomass.

Growth of young and old plants differs after clipping and nematode addition

In order to enhance insight in plant regrowth following herbivory, relative changes in plant biomass were recorded following clipping and nematode addition at variable plant ages. Relative shoot regrowth increased in response to defoliation and nematode addition, but the increase disappeared over time

(Figure 4.6). This temporal increase of shoot growth may be caused by a shoot sink created by defoliation. Following defoliation plants usually prioritize current photosynthates to shoots for compensatory growth (Harber et al. 1989), which can be constrained over time as photosynthesis capacity becomes restored and sink strength decays (Briske and Richards 1995). Defoliation at variable ages did not result in differences in shoot regrowth, although plants at these ages differed in sink strength at the time of defoliation because the amount of biomass removed increased with age at defoliation. Plant root growth was also temporarily increased by defoliation in young plants. This could be due to an increase of plant photosynthesis of the remaining foliage (Thomson et al. 2003) that directs recent photosynthate flow to roots (Schwachtje et al. 2006). Intermediate-aged plants were more constrained in photosynthesis than young plants, probably because more photosynthetic tissues were removed by defoliation causing stronger retardation of root growth and longer time taken before the root/shoot balance for optimal growth is regained (Johnson and Thornley 1987; Figure 4.6d). Relative root growth of old plants did not increase, indicating that limited amounts of assimilates of these plants were diverted to roots. Given that old plants suffered higher level of biomass removal in the current study, a higher sink priority may be given to regrow shoots in these plants and this relatively reduces sink strength in roots, preventing the increase of root growth. We suggest that shoot and root (re)growth responses after defoliation will differ and that responses depend on plant age at which defoliation takes place.

Conclusion

We have shown that the grass *H. lanatus* exhibits different growth and nutrient responses to defoliation and exposure to root-feeding nematodes and that these differences also depend on plant age of herbivory onset. Plants enhanced N concentration in foliage following nematode addition and defoliation in the short term, but reduced the N concentration of roots by nematode addition in the longer term. The changes in N concentration only occurred at specific plant ages. The plants tended to show highest relative regrowth of defoliated tissues shortly after defoliation and the relative

regrowth rate decreased over time independent of the plant age at which the defoliation occurred. On the other hand, relative root growth rate in defoliated plants was highly dependent on plant age at defoliation. Nematode addition only reduced plant root %N in the longer term and the reduction was not affected by plant age at which the nematodes were added.

Acknowledgement

Supplements

Table S4.1 Number of root-feeding nematodes in the nematode suspensions extracted at different times that were used for the inoculations.

Inoculum number	Extraction date	Mean[a]	SE
1	20-02-2014 13-03-2014	64	3.6
2	07-04-2014	109	17.1
3	28-04-2014	166	9.4

[a] indicates means of samples to estimate the total number of *P. penetrans* and *T. dubius* in each inoculum. Based on the total number of *P. penetrans* and *T. dubius*, the suspension was diluted or concentrated to achieve 100 individuals/5 ml for inoculation.
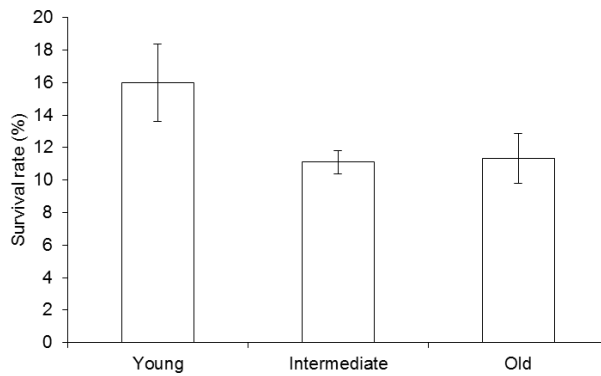

Figure S4.1 Survival rates of root-feeding nematodes (*P. penetrans* and *T. dubius*) one week after inoculation at young, intermediate, and old plant age. There were no significant differences between survival rates based on a one-way ANOVA test ($F_{2,25}$=2.77, *P*=0.082).
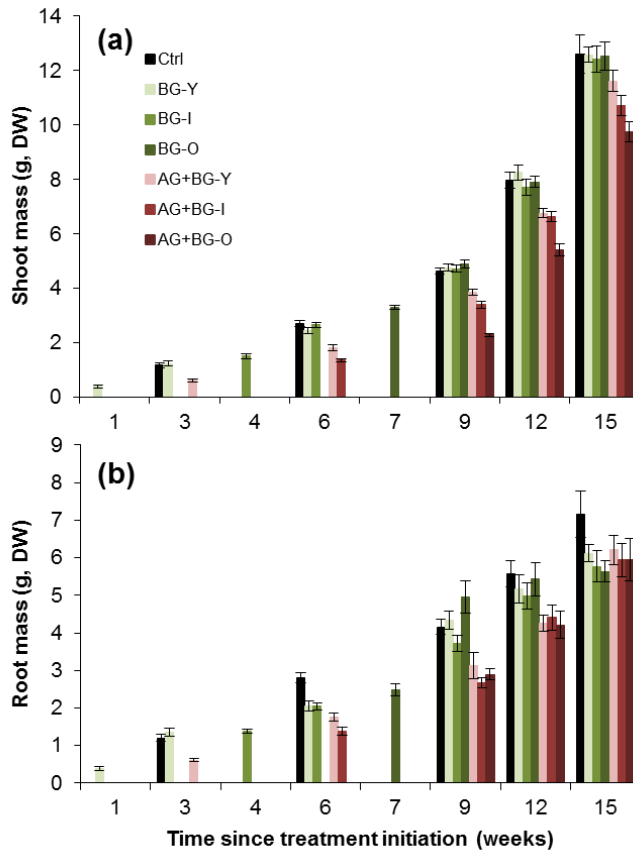
Figure S4.2 Temporal change of shoot (**a**) and root (**b**) biomass in plants exposed to nematodes (BG) nematodes and clipping (AG+BG) at young (Y, week 0), intermediate (I, week 3) or old (O, week 6) age. Plants were defoliated one week after each nematode inoculation at week 1, 4, 7, respectively. Control plants (Ctrl) were not exposed to AG or BG herbivory.
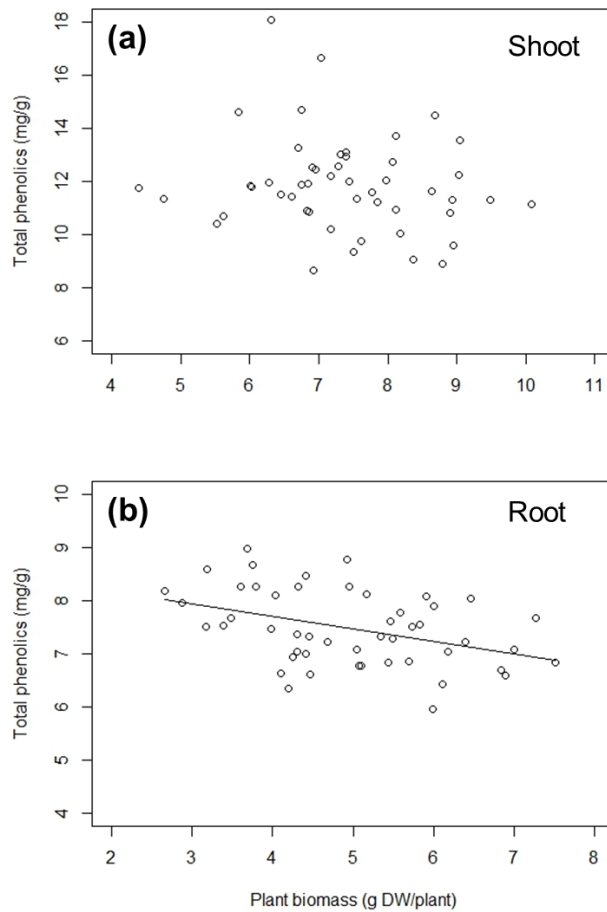
Figure S4.3 Relationship between plant shoot (a) and root (b) biomass (g dw per plant) and concentration of total phenolics in shoot or root tissues of *H. lanatus* at week 12. Each point represents an individual plant (n=49). The regression line indicates a significant relationship at *P = 0.05* level.

Chapter 5

Timing of simulated aboveground herbivory modifies population dynamics of root-feeding nematodes

Minggang Wang, T. Martijn Bezemer, Wim H. van der Putten, Arjen Biere and E. Pella Brinkman

To be submitted

Abstract

Plants are frequently damaged by both aboveground and belowground herbivores. Damage inflicted by aboveground herbivores can occur at different stages of plant development and can induce plant responses that affect the growth of belowground herbivores, however, little is known about effects of timing in this type of aboveground-belowground interaction. We tested how defoliations of the grass species *Holcus lanatus* during plant development influenced population development of root-feeding nematodes. We grew the grass in sterilized soil, inoculated with a mixture of two root-feeding nematode species, the endoparasite *Pratylenchus penetrans* and the ectoparasite *Tylenchorhynchus dubius*, and determined subsequent nematode population development. We defoliated the grass by clipping, which is not an exact mimicking of aboveground herbivory, but it enables analyzing how removal of photosynthetic tissues and redistribution from root to shoot may affect root-feeding nematode abundance and density. Plants were clipped either at 1, 4 or 7 weeks after inoculation. In general, defoliation increased the total abundance of *P. penetrans*, whereas both *P. penetrans* and *T. dubius* showed increased density, expressed as the number of nematodes per unit root mass. Timing of defoliation also influenced the density of *P. penetrans*, which increased only following early defoliation. Timing of defoliation did not influence *T. dubius* density. The proportion of *P. penetrans* in the roots compared to in soil decreased over time whereas the total number of *P. penetrans* in the pot did not change, suggesting that the suitability of roots for this nematode species decreased over time. The nitrogen concentration of plant roots was increased by defoliation, so that enhanced root quality may offer an explanation for the increase in abundance and density of root-feeding nematodes. Root biomass was reduced by defoliation and the reduction was strongest after early defoliation, which may have contributed to the stronger increase in *P. penetrans* density (number per unit root mass) after early than after late defoliation. We conclude that defoliation and the timing of defoliation can influence root-feeding nematodes, but that responses may be nematode species-specific. Responses of the nematode total numbers and densities may have been due to a combination of altered quality or quantity

of the host plant roots. Our results imply that timing will be a crucial factor in aboveground-belowground herbivore interactions.

Introduction

In grasslands, aboveground plant biomass is periodically removed by herbivore grazing or mowing. Defoliation may change plant nutrient content (Jaramillo and Detling et al. 1988) and plant productivity (McNaughton et al. 1998). As defoliation may change the availability of resources in roots and in the rhizosphere, it may also influence activities of decomposing microbes and root feeding soil fauna (Bardgett and Wardle 2003). Soil dwelling nematodes have frequently been used to test the response of soil biota to such aboveground disturbances (Bardgett 1997, Frank et al. 2000; De Deyn et al. 2004; Wang et al. 2006; Veen et al. 2010). Several studies reported declines in soil nematode densities following grazing (Todd 1996), which is expected to be due to reduced availability of belowground resources (Bardgett et al. 1997). However, other studies reported increases or non-significant effects of aboveground grazing by mammals or shoot clipping on the abundance of root-feeding nematodes (Freckman et al. 1979; Wall-Freckman and Huang 1998; Zolda 2006). The effects can be explained by changes in the quality and quantity of root substrate or by the reduction in plant resistance as a result of defoliation (Stanton 1988).

The majority of studies that examined effects of aboveground herbivory or grazing on soil dwelling nematodes focused on how defoliation affects root-feeding nematodes. Root-feeding nematodes directly interact with their host plants. They can act as important belowground pests in agricultural systems (Abawi and Widmer 2000). Several mechanisms have been proposed for the effects of grazing on root-feeding nematodes. Ingham and Detling (1984) proposed that grazing by prairie dogs increased the abundance of root-feeding nematodes due to a grazing-induced increase in favorable microclimate. Further, grazing can increase plant root growth, which could explain observed higher abundances of root-feeding nematodes (Schon et al. 2010). Finally, defoliation can also influence root-feeding nematode abundance by impacting on the nutritional quality of roots. For example, several studies have shown that aboveground defoliation can increase the nitrogen concentration of roots (Seastedt et al. 1988) or increase the carbon

fluxes to roots thereby influencing available resources to root-feeding nematodes and other soil organisms (Holland et al. 1996; De Deyn et al. 2004; Bazot et al. 2005). However, other studies have shown that defoliation can cause a decrease in nitrogen uptake of root feeders, possibly due to a decrease in plant root diameter (Mackie-Dawson 1999), or a decrease in nitrogen content in roots (Garcia and Rice 1994). This ultimately reduces the nutritional quality of the resources for root herbivores (Guitian and Bardgett 2000, Mikola et al. 2001, 2005; Lestienne et al. 2006). The ratio between carbon and nitrogen (C/N) in plant tissues is often considered an important measure of plant quality (Mattson 1980; Seastedt et al. 1988; Masters et al. 1993), and an increase in the C/N ratio in plants exposed to defoliation has been shown to correlate with a reduction in root-feeding nematode numbers (Bazot et al. 2005).

The impact of defoliation on plant-associated belowground processes and subsequently on belowground organisms can also depend on timing (Richards 1984; Hester et al. 2004; Ilmarinen et al. 2005). So far, the majority of studies on effects of timing of defoliation on plant responses have predominantly focused on aboveground plant responses (Maschinski and Whitham 1989; Obeso and Grubb 1994; García and Ehrlén 2002; Akiyama and Ågren 2012), whereas information on the responses of plant roots is scarce. Plants usually show seasonal changes in numerous properties, for example in traits related to resistance, levels of induced resistance (Karban and Baldwin 1997), plant chemistry (Bowers and Stamp 1993), or nutrient status (Mattson 1980). All these temporal changes may result in temporal responses of plant-associated organisms including root-feeding nematodes (Ilmarinen et al. 2005).

Plants also can show ontogeny-dependent growth responses to defoliation (McNaughton 1983; Boege 2005), which can influence root-feeding nematodes as well. For example, Ilmarinen et al. (2005) reported that defoliation reduced the root C/N ratio when plants were defoliated at an early growth stage, but increased the root C/N ratio when defoliation occurred at a later stage of plant development. In the same study, late defoliation promoted the abundance of herbivorous and predacious nematodes, whereas there was no such response

by other trophic groups of nematodes (Ilmarinen et al. 2005). Other studies have reported that aboveground herbivory can reduce the performance of belowground herbivores, but only when the aboveground herbivores arrive on the plant prior to the belowground herbivores (Erb et al. 2011; Johnson et al. 2012). The physiological responses of roots may also be determined by the timing of aboveground herbivory, which in turn may influence the performance of root-feeding herbivores.

Here, we examined how timing of defoliation of the grass *Holcus lanatus* influenced root-feeding nematode population dynamics in a mixture of *Pratylenchus penetrans* and *Tylenchorhynchus dubius*. We also examined how the carbon and nitrogen concentration in roots changed following defoliation. *Holcus lanatus* is a common perennial grassland species that occurs on various soil types (Jones and Turkington 1986). We focused on *P. penetrans*, because this endoparasitic species is commonly found in the roots of various grassland plant species (Thies et al. 1995) and is an economically important crop pathogen (Zunke 1990). The ectoparasite *T. dubius* is a common species that can develop high population densities and cause severe growth reduction to many plant species (Sharma 1971; Reynolds and Evans 1953). We tested the following hypotheses: 1) defoliation increases root quality (reducing C/N) and numbers of root-feeding nematodes. 2) Earlier defoliation more strongly increases root quality and root-feeding nematode populations than later defoliation. 3) Due to their different feeding location on plant roots (Klinkenberg 1963) *P. penetrans* (feeding on cortical cells) will respond more strongly to changes in root quality following defoliation than *T. dubius* (feeding on epidermal cells).

Materials and Methods

Soil, plant materials and inoculum

Soil was collected from a restored grassland (De Mossel, Ede, the Netherlands, 52.04 °N 5.44 °E) on former arable land. *Holcus lanatus* occurs abundantly in these restoration grasslands (Korthals et al. 2001, Kardol et al. 2005). In the

laboratory, the soil was sieved using 5 mm mesh, homogenized and gamma sterilized (> 25 KGray). Seeds of *H. lanatus* were obtained from a wild-seed supplier (Cruydt-hoeck, Nijeberkoop, The Netherlands). The seeds were surface sterilized with sodium hypochlorite (1%) for 1 minute and rinsed 4 times with demineralized water, sown on moist glass beads and placed in an incubator (16 h light, 25/20 °C day/night temperature) until germination.

Nematodes were collected from an agricultural field (Vredepeel, the Netherlands). The nematode community was dominated by two species of root-feeding nematodes: *P. penetrans* and *T. dubius*. These two species comprised 98.4 % of the root-feeding nematode community. The ratio of *P. penetrans* to *T. dubius* in the community was 10:1.

Experimental design

We filled 180 1 L pots each with 800 g soil (water content = 12.3% w/w) and planted one one-week-old seedling of *H. lanatus* in each pot. Pots were randomly placed in a greenhouse with ambient conditions of 16/8 h light/dark and 21/18±2 °C day/night. Three weeks later, all pots were inoculated with 4 ml nematode suspension containing on average 100 (SE = ± 6.8) nematode individuals per 4 ml (91 *P. penetrans* and 9 *T. dubius*). At week 0, nematodes were inoculated into two 1-cm-deep holes (2 ml per hole), which were closed immediately using the surface layer of soil. The soil surface of each pot was then covered with a thin layer of fine sand to minimize evaporation.

Plants were watered three times per week. Once a week, the soil moisture content was adjusted to 12.3% (w/w) with demineralized water and all the pots were rotated within the greenhouse to limit effects of position. Nutrients were added once per week using Hoagland solution (Hewitt 1966). The nutrient dosage was gradually increased over time to meet plant growth demands (Van der Putten et al. 1988), based on earlier measurements of C and N concentration of *H. lanatus* over time (T.M. Bezemer, unpublished data). A quarter-strength Hoagland solution was added in weeks 1-4 (from 12.5 ml to 50 ml per week in steps of 12.5 ml), half-strength solution was added in weeks

5-9 (from 60 ml to 100 ml per week in steps of 10 ml), and full-strength solution was added in weeks 10-15 (From 60 to 100 ml per week in steps of 10 ml). The experiment was carried out in a greenhouse at 70% relative humidity and a 16 h light (21°C) and 8 h dark (16°C) photoperiod regime. The natural daylight was supplemented with 400-W metal halide lamps when needed to insure a minimum of 225 µmol m^{-2} s^{-1} photosynthetically active radiation.

To examine effects of the timing of defoliation on nematode populations, four treatments were initiated and a subset of plants was harvested destructively every three weeks (Figure 5.1). For each treatment and harvest time there were 10 replicate pots. The sampling scheme included: (1) Early defoliation: fifty plants were defoliated one week after inoculation (week 1), and 10 randomly selected plants were harvested at each of the time points 3, 6, 9, 12 and 15 weeks. (2) Mid defoliation: forty plants were defoliated four weeks after inoculation (week 4), and 10 randomly selected plants were harvested at 6, 9, 12 and 15 weeks. (3) Late defoliation: thirty plants were defoliated seven weeks after inoculation (week 7) and 10 randomly selected plants were harvested at 9, 12 and 15 weeks after inoculation. (4) No defoliation: as a control, fifty plants were not defoliated and 10 randomly chosen plants were harvested at each of the time points at which plants of the early-defoliation treatment were harvested (Figure 5.1). One week after nematode inoculation, 10 non-defoliated plants were harvested to determine the number of nematodes that were recovered after inoculation. Plants were defoliated by clipping all leaves at 4 cm above soil surface using alcohol-sterilized scissors.

Plant harvest and nematode extraction

At harvest, the soil was rinsed off the roots of each plant in a bucket with tap water to achieve a 4-5 l suspension. The shoot was then separated from the roots by scissors and aboveground tissues were dried for at least 5 days at 40 °C before weighing. The suspension was stirred for 15 s and after letting the coarse soil particles settle for 30 s the water and suspended nematodes were decanted through a stack of 1 mm, 75 µm and three 45 µm sieves (Van der Stoel et al. 2002). The material from the 1 mm sieve was discarded and the

material from the 75 and 45 µm sieves was transferred to a double cotton milk filter (Hygia rapid, Hartmann AG, Herdenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink 1960). The nematodes were allowed to pass through the filter during 48 h at 20 °C, which delivered clean suspensions for nematode counting.

The migratory endoparasites (*P. penetrans)* were extracted from the roots using the funnel-spray method (Oostenbrink 1960) for 96 h and counted separately from the nematodes extracted from soil. The suspensions were stored at 4 °C until the nematodes were determined and counted at 50-200× magnification under an inverted light microscope. We identified and counted all the nematodes in each sample for the early harvests (until 9 weeks after inoculation), while for the last two harvests (weeks 12 and 15) subsamples of the soil samples were counted depending on the number of nematodes in the suspension.
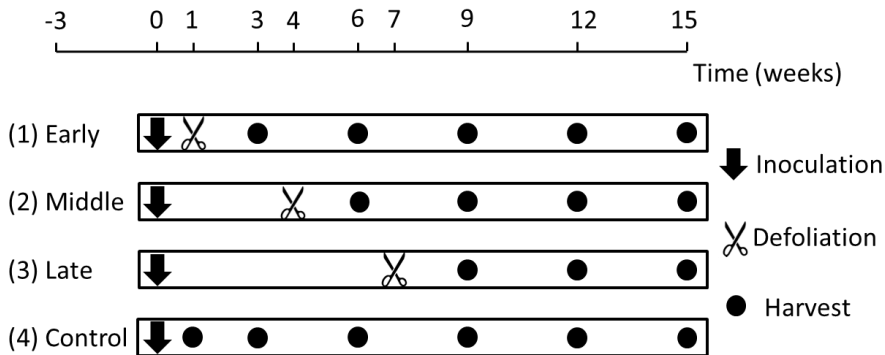

Figure 5.1 Experimental design: nematode inocula were introduced at T=0, which was three weeks after planting the grass in the pots. The arrows indicate the time point of inoculation, and scissor symbols indicate the time point of defoliation (1, 4 and 7 weeks after inoculation). The circles indicate the harvest time points of each treatment.

Plant carbon and nitrogen analysis

All the roots were oven-dried at 40 °C for a minimum of 5 days before weighing. Roots of 130 plants (10 replicates harvested at 3, 6, 9 and 12 weeks

after nematode inoculation for the control and early defoliation treatments; 6, 9 and 12 weeks after inoculation for the mid defoliation treatment, and 9 and 12 weeks after inoculation for the late defoliation treatment) were ground to a powder and 1 mg was weighed into tin capsules. Carbon (C) and nitrogen (N) concentrations were measured using a C/N analyzer (Flash EA 1112, Interscience, Breda, NL).

Data analysis

We recorded the number of nematodes in plant roots and soil in each pot and calculated total number and density (total number of nematodes per gram dry root biomass) per pot. This was done separately for *P. penetrans* and *T. dubius*. We randomly allocated subsets of the ten replicates of the non-defoliated plants that were harvested at 6, 9 and 12 weeks after inoculation to each of the defoliation treatments (early, mid and late), in order to obtain at least 3 non-defoliated control replicates for each time point and for each defoliation treatment. Thereafter, we performed a three-way ANOVA with defoliation (Defo: +/-), timing of defoliation (Timing: early, mid, and late), and weeks after defoliation (Weeks: 2, 5 and 8 weeks after defoliation) as fixed factors. We were particularly interested in testing the impact of defoliation (factor "Defo") and its interactions with the factor "Timing" and "Weeks". The interaction between "Defo" and "Timing" tests whether the impact of defoliation on nematode population depends on the timing of defoliation (early, mid and late). The interaction between "Defo" and "Weeks" tests whether the time-course of population development in the eight weeks following defoliation is different from that on non-defoliated plants. The interaction between "Defo", "Timing" and "Weeks" tests whether defoliation-induced changes in the time course of population development in the eight weeks following defoliation depend on when the plants were defoliated. To examine whether root quality was affected by defoliation and its timing, we analyzed root C and N concentration and C/N ratio using the three-way ANOVA previously described. As we did not measure the root resources of plants of the last harvest (15 weeks after nematode inoculation), only two levels (3 and 6 weeks after inoculation) were included in the analysis. Root biomass of plants was

also analyzed using the three-way ANOVA described above to test whether and how the timing of defoliation influenced plant biomass production. Tukey *post-hoc* tests (P < 0.05) were performed to compare treatment levels within each significant main factor.

Data of nematode numbers and density were Log_{10} (x+1) transformed and root biomass was Log_{10} (x) transformed prior to analyses to meet the assumption of homogeneity of variances.

Results

Nematodes

Density Defoliation increased the density of *P. penetrans* relative to the non-defoliated controls, but only when plants were defoliated early (Defo × Timing interaction, Table 5.1, Figure 5.2a, 5.2c and 5.2e). The extent to which defoliation increased *P. penetrans* density decreased over time (Weeks × Defo, Table 5.1, Figure 5.2a, 5.2c and 5.2e). Defoliation also significantly increased the density of *T. dubius*, but this was independent of the timing when the defoliation occurred (main effect of "Defo", Table 5.1, Figure 5.2b, 5.2d and 5.2f).

Total number The total numbers of *P. penetrans* per pot only marginally increased over time, but were significantly enhanced by defoliation during the period of study (main effect of "Defo", Table 5.2, Figure 5.3a, 5.3c and 5.3e). By contrast, the total number of *T. dubius* strongly increased during the period of study (main effect of "Weeks"), whereas their numbers were neither significantly affected by defoliation, nor by the timing of defoliation within the timeframe of study (Table 5.2, Figure 5.3b, 5.3d and 5.3f).

Proportion of P. penetrans in roots The proportion of *P. penetrans* inside roots decreased over time within the examined timeframe (main effect of "Weeks"), but this was not influenced by defoliation (Table 5.3, Figure 5.4).

Table 5.1 ANOVA of the density of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* (numbers of individuals per gram root) extracted from roots or in soil 2, 5 or 8 weeks after defoliation. The host plant *H. lanatus* was exposed to early, mid or late defoliation.

Sources	df	P. penetrans		T. dubius	
		F	P	F	p
Weeks	2	25.38	**< 0.001**	10.90	**< 0.001**
Defo	1	22.49	**< 0.001**	7.30	**0.008**
Timing	2	8.31	**< 0.001**	5.80	**0.004**
Weeks × Defo	2	4.04	**0.020**	0.65	0.524
Weeks × Timing	4	4.63	**0.002**	1.08	0.371
Defo × Timing	2	3.23	**0.043**	0.23	0.793
Weeks × Defo × Timing	4	0.57	0.685	0.54	0.710
Error	121/119[*]				

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (Defo, +/-) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate significance at level $P < 0.05$.

[*] 121 and 119 indicate the *degree of freedom* of the error term for *P. penetrans* and *T. dubius* in the three-way ANOVA, respectively.

Root biomass

Root biomass was greatly reduced by defoliation during the timeframe analyzed. The extent of this reduction was smaller in the early and late defoliated plants than in the plants defoliated mid way (significant Defo × Timing interaction) and varied with time after defoliation (significant Defo × Weeks interaction, Table 5.4, Figure 5.5).

Root C, N concentration

C and N concentration of roots followed different ontogenetic patterns (Figure 5.6). Whereas root N concentrations linearly decreased over time, root C concentration peaked after 6 weeks of growth and decreased afterwards. Defoliation did not significantly affect root C concentrations or C/N ratio during the examined five weeks following defoliation, while root N concentrations were enhanced by defoliation (main effect of "Defo", Table 5.5, Figure 5.6).
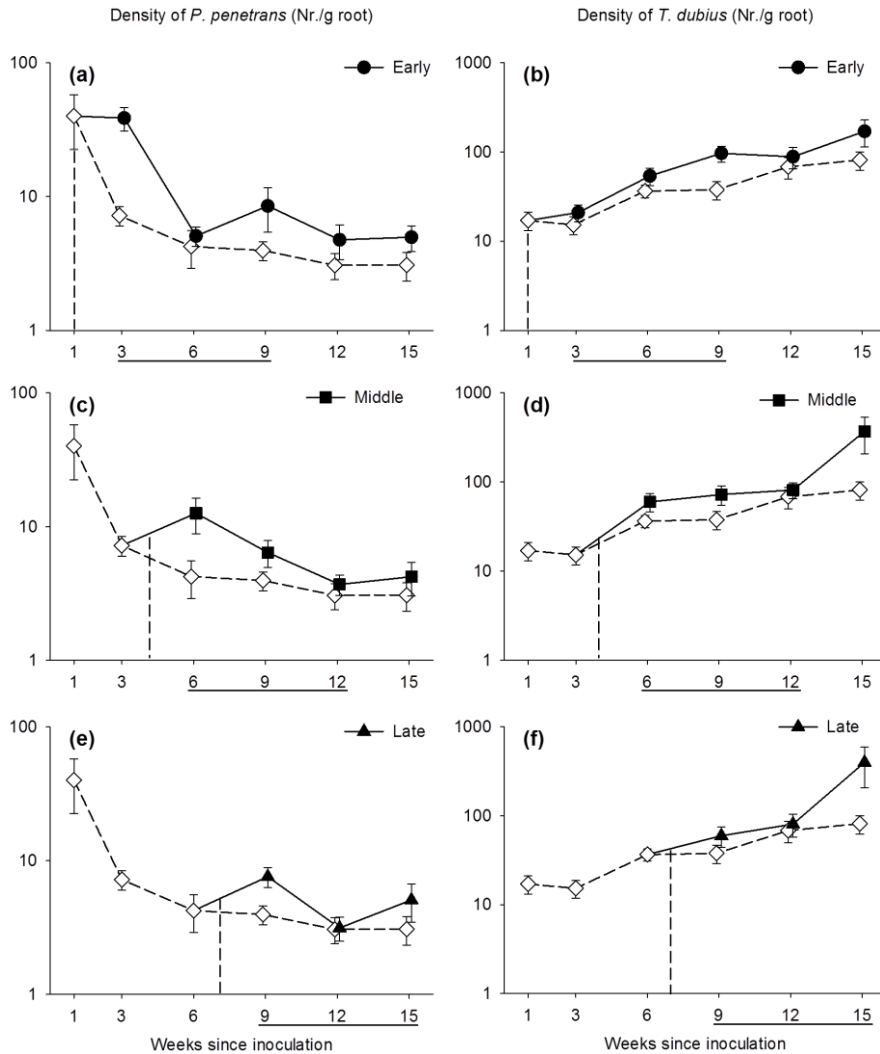
Figure 5.2 Mean (±SE) density of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* in each pot when their host plant *Holcus lanatus* was defoliated (filled symbols) or not defoliated (open diamonds). Plants defoliated at week 1 (filled circle), 4 (filled square) or 7 (filled triangle) after nematode inoculation were regarded as early (**a**, **b**), middle (**c, d**) and late (**e, f**) defoliation, respectively. The vertical dashed line indicates the time of defoliations. Data of harvest at underlined weeks was used for statistical analysis. See table 5.1 for statistics.

Table 5.2 ANOVA of the total number of *P. penetrans* and *T. dubius* in pots 2, 5 or 8 weeks after defoliation. The host plant *H. lanatus* was exposed to early, mid or late defoliation.

Sources	df	P. penetrans		T. dubius	
		F	p	F	p
Weeks	2	1.88	0.157	53.17	**< 0.001**
Defo	1	6.65	**0.011**	1.47	0.227
Timing	2	2.56	0.082	35.28	**< 0.001**
Weeks × Defo	2	1.55	0.216	1.85	0.161
Weeks × Timing	4	2.44	0.051	2.26	0.067
Defo × Timing	2	0.70	0.500	0.01	0.990
Weeks × Defo × Timing	4	0.79	0.536	0.72	0.580
Error	121/119[*]				

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (Defo, +/-) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate the significances at level $P < 0.05$.

[*] 121 and 119 indicate the *degree of freedom* of the error term for *P. penetrans* and *T. dubius* in the three-way ANOVA analysis, respectively.

Discussion

General effects

Our study shows that defoliation increased both the population size and density of the migratory endoparasitic nematode species *P. penetrans* relative to their non-defoliated controls. However, in the case of the ectoparasitic nematode species *T. dubius* defoliation only increased its abundance per gram root, and not the total population size per pot, indicating specific responses of nematode species. Defoliation enhanced plant N concentration but reduced plant root mass, suggesting that the changes in population size and density of the root-feeding nematodes following defoliation may represent a response to both altered quality and quantity of roots. Whether these differences are species-specific or whether they reflect differences related to their feeding types would require additional work including multiple species per feeding type. The effect of defoliation on the density of *P. penetrans* depended on the timing of defoliation. Effects of defoliation on root-feeding nematodes have been previously demonstrated however, hitherto there has been little evidence how timing of defoliation may affect the outcome of this type of above-belowground interaction.

Responses of root-feeding nematodes to timing of defoliation

Defoliation increased the abundance of *P. penetrans* relative to the non-defoliated controls, which supports part of our first hypothesis. This increase may have been caused by the higher root N concentration as a result of defoliation (Todd 1996). It suggests that the population growth of *P. penetrans* to defoliation was modified by root quality. This result is in line with a study that reported a decreased root C/N ratio causing an increase in root-feeding nematode abundance as a result of defoliation (Bazot et al. 2005). However, in that study no increase in root N concentration was observed in response to defoliation, illustrating that plant species may differ in their response to defoliation (Guitian and Bardgett 2000). In contrast to *P. penetrans*, the abundance of *T. dubius* did not respond to defoliation in the current study (Figure 5.3), indicating that nematode species can also differ in their responses to changes in plant quality. It may be that our analysis of total N in the root material does not reflect changes in N concentration among feeding sites, which is mostly phloem for *P. penetrans* and outer cortical cells for *T. dubius* (Perry and Wright 1998). Future studies should examine how changes in actual plant quality of the feeding sites may influence different nematode species and how this influences interspecific competition between nematodes (Brinkman et al. 2005).

In contrast to the total number per pot, the number of root-feeding nematodes per unit root mass (density of nematodes) was enhanced by defoliation for both nematode species. As the total abundance of *T. dubius* was not affected by defoliation, this shows that the enhanced density of *T. dubius* following defoliation can be explained by the less rapid increase in root biomass following defoliation. On the other hand, the increased density of *P. penetrans* could be caused by both enhanced nematode abundance and less rapid increase in root biomass following defoliation (Tables 5.2 and 5.4). Moreover, the density of *T. dubius* steadily increased during the examined period with plant root biomass being also increased, and this again indicates that the

growth rate of *T. dubius* population proceeded faster than the growth rate of root biomass.
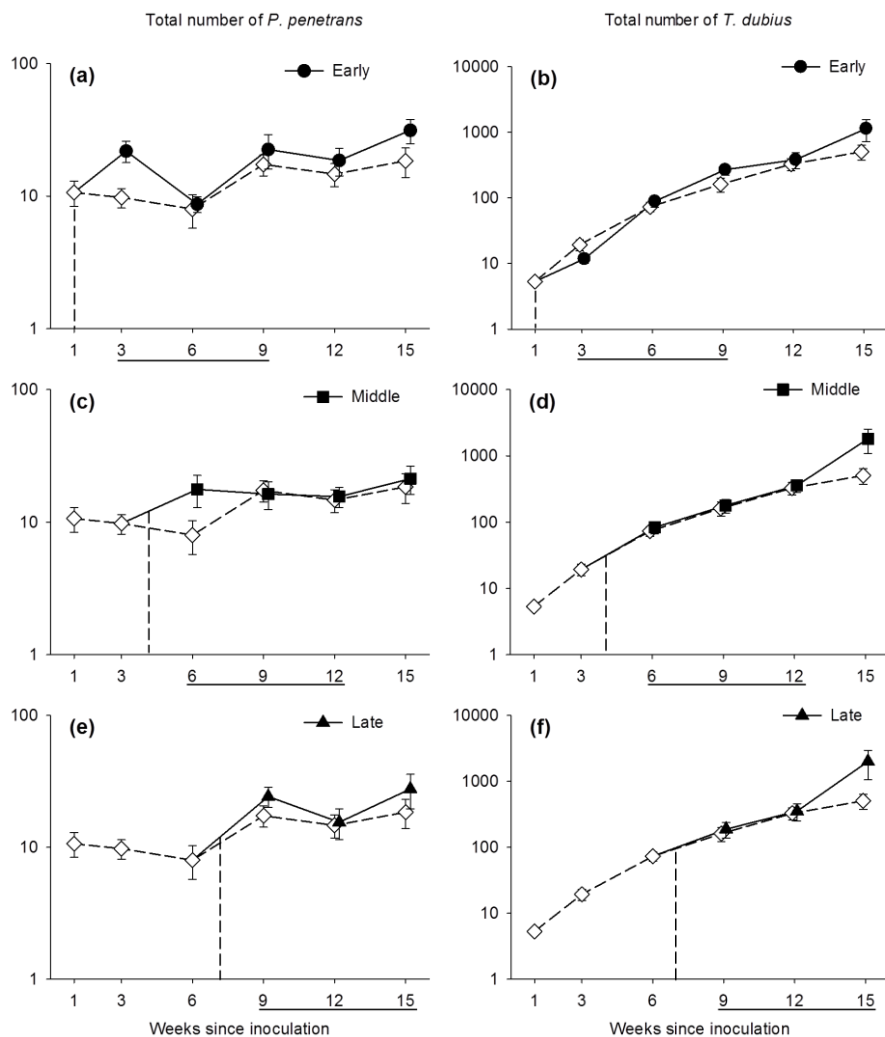

Figure 5.3 Mean (±SE) total number of *Pratylenchus penetrans* and *Tylenchorhynchus dubius* per pot when their host plant *Holcus lanatus* was defoliated (filled symbols) or not defoliated (open diamonds). Plants were defoliated at week 1 (filled circle), 4 (filled square) or 7 (filled triangle) since addition of nematodes to 3 weeks-old plants, regarded as early (**a**, **b**), middle (**c**, **d**) and late (**e**, **f**) defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvests at underlined weeks were used for statistical analysis. See Table 5.2 for statistics.

Table 5.3 ANOVA results on proportion of total *P. penetrans* present in roots of host plant *H. lanatus* 2, 5 or 8 weeks after defoliation. The plants were exposed to early, middle or late defoliation

Sources[a]	df	Proportion[b]	
		F	p
Weeks	2	6.50	**0.002**
Defo	1	0.07	0.800
Timing	2	2.30	0.105
Weeks × Defo	2	1.12	0.330
Weeks × Timing	4	2.76	**0.031**
Defo × Timing	2	0.39	0.677
Weeks × Defo × Timing	4	1.97	0.103
Error	120		

[a]Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (Defo, +/-) and the timing of defoliation (Timing, Early/mid/late) as the main factors. Bold values indicate significance at level $P < 0.05$.

[b]The proportion was calculated as number of *P. penetrans* extracted from roots divided by total number of *P. penetrans* extracted from both roots and soil in a pot.

We hypothesized that the changes in total population sizes of root-feeding nematodes following defoliation will depend on the timing of defoliation. In contrast to this hypothesis, we did not observe changes in total population size of either nematode species in response to the timing of defoliation (Table 5.2, Defo×Timing interaction). Nevertheless, we did observe an increase in the density of one of the two nematode species, *P. penetrans*, which only occurred following early defoliation, suggesting a dependence of defoliation effects on plant development stage. We hypothesized that effects of the timing of defoliation should be caused by growth stage-specific changes in plant quality following defoliation as suggested by Ilmarinen et al. (2005). Unexpectedly, overall root quality, as indicated by N concentration was not influenced by the timing of defoliation in our study, suggesting that root quality did not change with time at which defoliations occurred. However, because *P. penetrans* feeds on phloem and *T. dubius* feeds on cell contents in roots, an overall root N concentration may not represent root quality available to these nematodes. An examination on root quality of these feeding sites is needed to better predict the population dynamics of each nematode species. Since no effect of the timing of defoliation was observed on the total abundance of *P. penetrans*, it is likely that the effect of timing of defoliation on the density (number per unit root mass) of *P. penetrans* was mediated by a less strong

reduction in root biomass following early than late defoliation, although the strongest reduction seemed to occur at the mid defoliation (Figure 5.5). Therefore, we suggest that the timing of defoliation may also determine the nematode response to defoliation because the proportion of nematodes per unit root mass increases due to retardation of root growth during the recovery from defoliation. On the other hand, neither abundance nor density of *T. dubius* was influenced by the timing of defoliation, which is consistent with our hypothesis that ectoparasitic nematode species are less sensitive to changes in root quality and/or quantity than endoparasites.

Pratylenchus penetrans is a migratory endoparasitic nematode that can occur either inside or outside root tissues, and whose choice between root and soil may indirectly reflect how favorable the conditions of root tissues are (Zunke 1990). Although defoliation did not impact the proportion of *P. penetrans* in roots, the location preferences of *P. penetrans* appeared to change during plant development. A relatively higher proportion of *P. penetrans* was extracted from root tissues of plants at the time during early defoliation, indicating that at this time point *P. penetrans* prefers remaining inside the roots rather than moving out. This observation corresponds with the changes in plant quality (C/N ratio) in roots after defoliations: the proportion of *P. penetrans* inside roots decreased as root quality decreased over time. As we could not relate the proportion of root *P. penetrans* to root N concentration over time (Regression analysis: $R^2 = 0.04$), our data suggest that other attributes in plant roots changed during plant development leading to the decreased proportion of *P. penetrans* in roots. One possibility may be that secondary chemicals may accumulate when roots age (Elger et al. 2009; Quintero and Bowers 2012).

Responses of root biomass

It has been argued that plants can allocate more resources to roots after defoliation (Hokka et al. 2004; Ilmarinen et al. 2005). However, in our study we did not observe an increase of root biomass in response to defoliation. In contrast, we observed that root biomass was reduced by defoliation and that

the reduction alleviated during regrowth (Figure 5.5). Grass species can greatly differ in how they reallocate resources between shoot and root
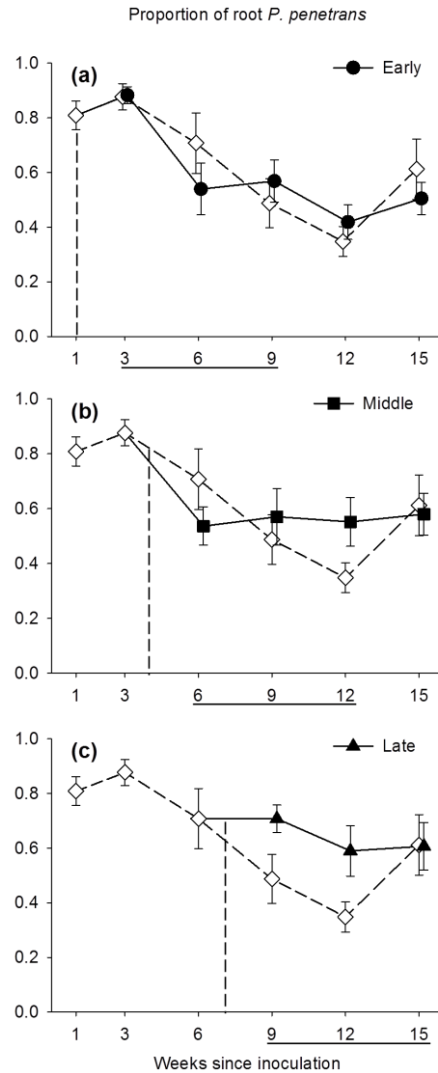

Figure 5.4 Mean (±SE) proportion of *Pratylenchus penetrans* numbers that were extracted from roots divided by the total number in each pot of *Holcus lanatus* that was defoliated (filled symbols) or not defoliated (open diamonds). Plants defoliated at week 1 (filled circle), 4 (filled square) or 7 (filled triangle) after inoculation were regarded as early (**a**), middle (**b**) and late (**c**) defoliation, respectively. The vertical dashed line indicates the time of defoliation. Data of harvests at underlined weeks were used for statistical analysis. See table 5.3 for statistics.

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components following defoliation (Wilsey et al. 1997). In our study the defoliation-induced reduction of root biomass suggests that plants allocated resources to shoot regrowth at the expense of root biomass. In addition, plants typically change their priority of resource allocation to vegetative growth compared to storage or reproductive demands during development (Boege and Marquis 2005), which may also contribute to plant biomass responses to defoliation over time. However, in the current study, we used a perennial grass species and the defoliation treatments were applied during the vegetative growth phase of the plant. Thus, the possibility that the root biomass response to defoliation was the result of a priority switch towards allocation to reproduction can be excluded. Instead, the plant biomass response to defoliation observed in our study was mainly determined by the growth phase at defoliation either via compensating defoliated tissues or via allocating resources to roots. The reduced root biomass and unaltered C concentration in roots of defoliated plants suggests that regrowth of shoot biomass of *H. lanatus* may have been prioritized in terms of resource allocation rather than resource storage in roots in this study.

Table 5.4 ANOVA of plant root biomass of the host plant *H. lanatus* 2, 5 or 8 weeks after defoliation. Plants were exposed to early, middle or late defoliation.

Sources	df	Root	
		F	P
Weeks	2	297.18	**< 0.001**
Defo	1	48.89	**< 0.001**
Timing	2	219.93	**< 0.001**
Weeks × Defo	2	7.11	**0.001**
Weeks × Timing	4	17.80	**< 0.001**
Defo × Timing	2	6.89	**0.001**
Weeks × Defo × Timing	4	2.34	0.060
Error	122		

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (Defo, +/-) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate significance at level $P < 0.05$.
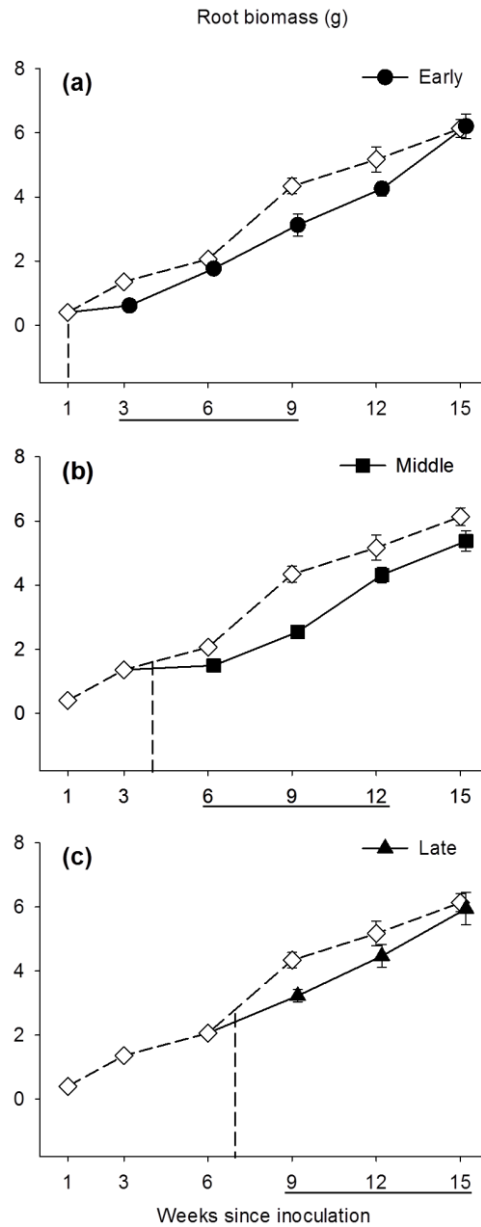
Figure 5.5 Mean (±SE) root biomass of *Holcus lanatus* that were exposed to defoliation (filled symbols) or no defoliation (open diamonds). Plants defoliated at week 1 (filled circle), 4 (filled square) or 7 (filled triangle) after inoculation were regarded as early (**a**), middle (**b**) and late (**c**) defoliation, respectively. Data of harvests at underlined weeks were used for statistical analysis. See table 5.4 for statistics.

Responses of plant quality to timing of defoliation

In general, during their life plants change their resource allocation to maintain functional priorities (Boege and Marquis 2005). In our study, we expected that root C concentration should have been reduced to meet the demands of shoot regrowth (Kursar and Coley 2003), whereas the root N concentration should be enhanced due to improved N availability (Holland and Delting 1990) as a result of defoliation. Surprisingly, we did not observe effects of defoliation on C concentration in roots, suggesting that defoliation did not result in a net carbon flow from root to shoot. This may be because the regrowth of shoot tissues was based on assimilates produced from remaining shoot tissues (Briske and Richards 1995) and hence no root resources were needed. Nevertheless, as expected (Seastedt et al. 1988; Green and Detling 2000; Hokka et al. 2004), defoliation increased plant N concentration in the roots.

Table 5.5 ANOVA of carbon (C) and nitrogen (N) concentration and C/N ratio in the roots of *H. lanatus* 2 or 5 weeks after defoliation. Plants were exposed to early, middle or late defoliation.

Source	df	C (%)		N (%)		C/N	
		F	p	F	p	F	p
Weeks	2	5.16	**0.026**	2.38	0.126	9.32	**0.003**
Defo	1	0.36	0.549	11.62	**< 0.001**	3.92	0.051
Timing	2	13.64	**< 0.001**	5.28	**0.006**	14.29	**< 0.001**
Weeks × Defo	2	0.52	0.474	0.16	0.691	0.81	0.372
Weeks × Timing	4	8.18	**< 0.001**	1.27	0.285	12.09	**< 0.001**
Defo × Timing	2	0.78	0.462	0.88	0.420	1.24	0.294
Weeks × Defo ×Timing	4	1.35	0.264	1.06	0.350	0.58	0.560
Error	88						

Data were analyzed by 3-way ANOVA: Weeks after defoliation (Weeks, 2/5/8 weeks), defoliation (Defo, +/-) and the timing of defoliation (Timing, Early/mid/late) were the main factors. Bold values indicate significance at level $P < 0.05$.

This increase can be caused by a temporal accumulation of N in plant roots due to reduced transport to defoliated aerial tissues. Other studies reported a decrease of root quality following defoliation because N was transported from roots to shoots for compensational regrowth (McNaughton 1983; Augustine and McNaughton 1998). These mixed results may depend on which plant

species is being defoliated, as plant species can differ greatly in their tolerance or defense strategies to tissue losses (Damhoureyeh and Hartnett 2002; Hokka et al. 2004; Del-Val and Crawley 2005). Opposite to the higher N concentration in roots when *Plantago* species were defoliated at an early stage during the growing season (Ilmarinen et al. 2005), our study did not witness a timing effect of defoliation (Defo×Timing) on root N concentration (Figure 5.6), which suggests *H. lanatus* always prioritizes N flow to roots regardless of the timing of defoliation.
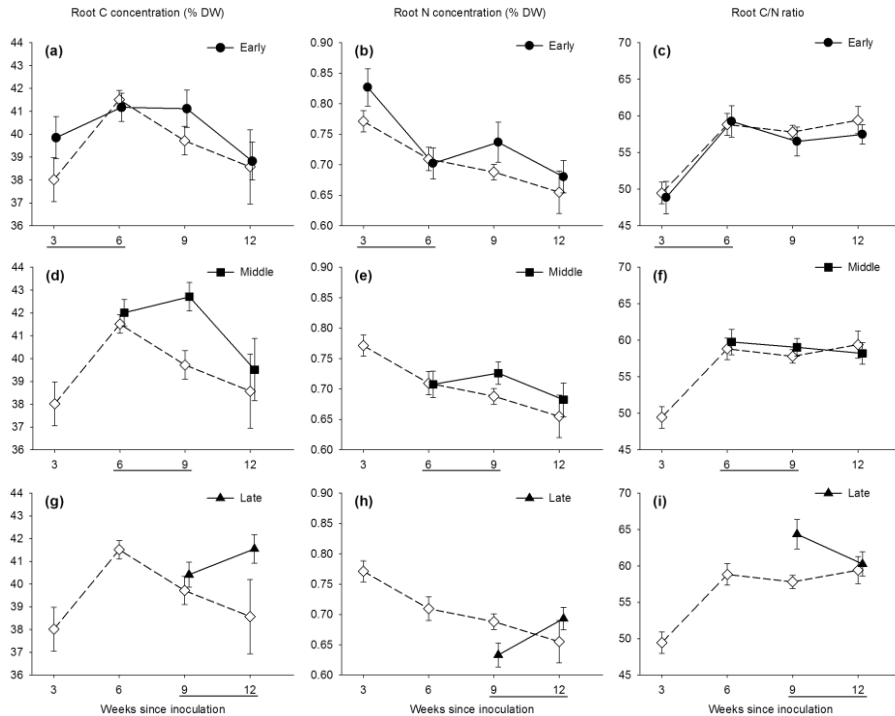

Figure 5.6 Mean (±SE) root carbon and nitrogen concentration and C/N ratio of *Holcus lanatus* that were exposed to defoliation (filled symbols) or no defoliation (open diamonds). Plants defoliated at week 1 (filled circle), 4 (filled square) or 7 (filled triangle) after nematode inoculation were regarded as early (**a**, **b** and **c**), middle (**d**, **e** and **f**) and late (**g**, **h** and **i**) defoliation, respectively. Data of harvests at underlined weeks were used for statistical analysis. See table 5.5 for statistics.

Conclusion

We conclude that defoliation increased the abundance and density of *P. penetrans* but only increased the density of *T. dubius*, indicating a species-specific response of root-feeding nematodes to the same events of defoliation (Wondafrash et al. 2013). Further, our study indicates that only defoliation that occurs soon after nematode inoculation can cause an increase in the density of *P. penetrans*, pointing at the possible significance of timing of defoliation in above-belowground interactions. The analysis of plant quality and biomass indicates that *P. penetrans* may be more sensitive to quality alteration, while the species *T. dubius* may be more responsive to changes in root quantity. Our study highlights the importance of considering the timing of defoliations, the specific responses of herbivores to these defoliations, and the consequences for the proportion of nematodes per unit of root biomass in above-belowground herbivore interactions.

Acknowledgements

Chapter 6

General discussion

Aims and outline

In this thesis, I investigated above-belowground herbivore interactions across variable time scales of herbivory. In chapter 2 I reported that the sequence of when the above- and belowground herbivores arrived on the plant significantly affected these interactions. I also highlighted the importance of prior aboveground herbivory for the performance of later arriving conspecifics. Consequently in chapter 3 I exposed plants to aboveground herbivory across a time series prior to later arriving conspecifics to examine the responses of conspecific herbivores. In this chapter I further examined if these responses depend on root colonization by arbuscular mycorrhizal fungi (AMF). In both chapters I showed the importance of induced plant defense in mediating interactions between AG and BG herbivores as well as symbionts. In addition to induced defenses that are used by many plant species to defend themselves against herbivory, plant species may also tolerate damage of these herbivores (Agrawal 2000b). Therefore in chapter 4 I tested whether AG damage and BG herbivory during plant ontogeny can modify both plant tolerance and induced defenses. The results suggest that the age at which the plant experiences AG herbivory determines how it shapes plant root growth but not induced root defense. Subsequently, I recorded the population dynamics of root herbivores in plants defoliated at different plant ages in chapter 5. In this chapter I discuss the main findings of this thesis, point out limitations of these studies, and propose several future directions in studies of temporal aspects of above-belowground interactions.

Timing of attack by aboveground and belowground herbivores on plant induced defense

Plants can be attacked by aboveground (AG) and belowground (BG) herbivores at any time as they develop. As plant responses to early herbivore attack can modify the performance of secondarily attacking herbivores (Karban and Baldwin 1997), timing of arrival on host plants is an important aspect influencing both inter- and intraspecific herbivore interactions (Tiffin 2002). Some studies have reported that early arriving herbivores can strongly

reduce the performance of later arriving herbivores (Viswanathan et al. 2007), while attacks by secondary herbivores did not impact herbivores that were there first, or even at the same time (Viswanathan et al. 2007; Poelman et al. 2008). Given that AG and BG herbivores can also systemically influence each other, I hypothesized that whether herbivores arrive early or late on plants will also matter in AG-BG herbivore interactions (see Erb et al. 2011; Johnson et al. 2012).

In chapter 2 I introduced AG and BG herbivores in different sequences and recorded the performance of plants and herbivores. By calculating herbivore weight gain over a period of 17 days, I showed that when the AG herbivore preceded the BG herbivore in arriving at the plant *Plantago lanceolata*, it tended to facilitate BG herbivore growth (Figure 2.2). This result contrasts with many other studies reporting reductions of BG herbivore performance in leaf-damaged plants (e.g. Tindall and Stout 2001; Soler et al. 2007). In wild and cultivated maize plants Erb et al. (2011) showed that only prior but not simultaneously or later arriving AG herbivores can reduce the colonization by root-feeding larvae and they proposed that these arrival-sequence-specific effects should be caused by an increase in secondary metabolites that were systemically induced by AG herbivory. I therefore measured the level of Iridoid glycosides (IGs), the major secondary defense compounds in *P. lanceolata* plants (Bowers and Puttick 1989) in the different sequential AG-BG treatments. I hypothesized that the facilitation of BG herbivore growth by earlier arriving AG herbivores could be caused by a decrease of IGs in plant roots. However, the concentration of root IGs in *P. lanceolata* was not altered by AG herbivory in this study. Since the composition of IGs in *P. lanceolata* can also affect herbivore performance (Bennett et al. 2013), I further measured the relative concentration of aucubin and catalpol, the main IGs in *Plantago spp*. I expected that AG herbivory shifted the composition of IGs, e.g. by increasing the proportion of aucubin relative to the more toxic catalpol and that this would consequently benefit the BG herbivores. However, I did not observe the expected shift in composition of root IGs in this study, although other studies suggest that the metabolic profile of *P. lanceolata* can be critical in interpreting the outcome of AG-BG herbivore interactions (Sutter and Müller

2011). In other plant species, shifts in the composition of defense compounds upon herbivory and their importance in plant-herbivore interactions were also reported. For example, a recent study reported an altered pattern in soluble free and soluble conjugated phenolic acids in leaf-damaged maize (*Zea mays*) roots was responsible for reduced BG herbivore growth (Erb et al. 2015). In my research AG herbivory did not significantly alter the level of root IGs but BG herbivory reduced it, so I concluded that the facilitation of BG herbivores could result from a lower level of root defense compounds due to inhibition by the BG herbivores themselves (Figure 2.3). This conclusion awaits further tests, yet it paves an avenue for studying plant-herbivore relations within the context of intraspecific competition of herbivores.

Exposure of plants to variable sequences of AG and BG herbivores was also performed to unravel the effects of relative sequence of arrival of these herbivores on the performance of later arriving AG herbivores. BG herbivores, in the absence of aboveground herbivores, tended to reduce feeding consumption of later arriving AG herbivores, but this was independent of the sequence of BG herbivory relative to the arrival of the AG herbivore. It suggests that BG herbivory reduced the feeding consumption of AG herbivores regardless of the timing of herbivory. A major finding in this study is that AG herbivores can reduce the growth of their conspecifics only when they colonized plants before the conspecifics arrived on the plant. Similar sequence-specific effects have been reported previously (Viswanathan et al. 2007; Poelman et al. 2008; Gomez et al. 2012). In contrast to these studies that mostly reported changes in concentrations of defense compounds or primary assimilates, I did not observe altered concentrations of IGs by AG herbivory in plant foliage (Figure 2.3). The reduction of AG herbivore growth should be attributed to other potentially induced defense traits, lower foliar nutrient values or physical defenses, which directly reduced leaf consumption by these herbivores. A shift in the relative composition of defense compounds can also lead to significant responses of herbivores (Bennett et al. 2013), although it was not found in the current study (chapter 3). Another major finding in this thesis is that I observed that induced defense by AG herbivory in plant foliage against later conspecifics was canceled out when the plant roots were also

exposed to feeding by BG herbivores (Figure 2.3). It shows that the details of the damage the plant experiences, including the site of damage and when damage occurs, and the identity of the herbivore, can greatly determine imminent herbivory events and herbivore population growth in the longer run.

Time course of induced defense by AG herbivores

Plant defenses are energy costly but can benefit plants under herbivory (Baldwin 1998) and the level of defense is an important determinant of plant fitness. The levels of herbivory that a plant experiences can vary greatly over time and induced defenses should only be expressed at high risk of attack. Low levels of induction can save the plant energy that can be used for growth or maintenance (Karban and Baldwin 1997) in situations when herbivores are absent or negligible (Adler and Karban 1994). To maximize fitness, plants usually show a temporal pattern in magnitude of induction following herbivory, depending on the level of herbivory encountered. Plant defenses induced by herbivory usually increase immediately after the occurrence of herbivory, level off and then decline until the pre-herbivory level or even lower than that level (Figure 6.1). I investigated the time course of induced defense by AG caterpillars against their conspecifics in Chapter 3 because we observed a negative effect of AG herbivory on the performance of later arriving conspecific AG herbivores in a previous study (chapter 2).

The time lag between damage and the onset of defense and between cessation of damage and relaxation of defense can be crucial in the effectiveness of induced defense for deterring herbivory (Karban 2011). Few studies have recorded the time course of induced defense, but these studies show that it can be expressed within several hours after herbivory (Hopkins et al. 2009) but also that it can take more than 28 days after a herbivory event before defense levels start to decline (Gomez et al. 2010). Mathur et al. (2011) found that glucosinolates, defense compounds in *Brassica juncea*, were systemically induced 4 days after foliar damage by *Spodoptera* spp. caterpillars. The level of glucosinolates remained high for 7 days and subsided 14 days after

herbivory damage. In this thesis I used the AG herbivore *Spodoptera exigua* and the plant species *P. lanceolata* and hypothesized that plant defense would increase over a time series of 1, 2, 4 and 8 days after induction (chapter 3). The results support this hypothesis by showing a significant increase of plant defense (measured as herbivore growth) over time after herbivory induction (Figure 3.2). The decrease in herbivore performance corresponded with a gradual increase in the level of the defense compound catalpol in plant foliage in the 8 days following induction. However, the consumption rate by the herbivores did not change over time. Thus these results reflected that the efficiency of food conversion to body biomass in the herbivore was reduced, indicating an induced decrease of food quality by prior conspecifics.
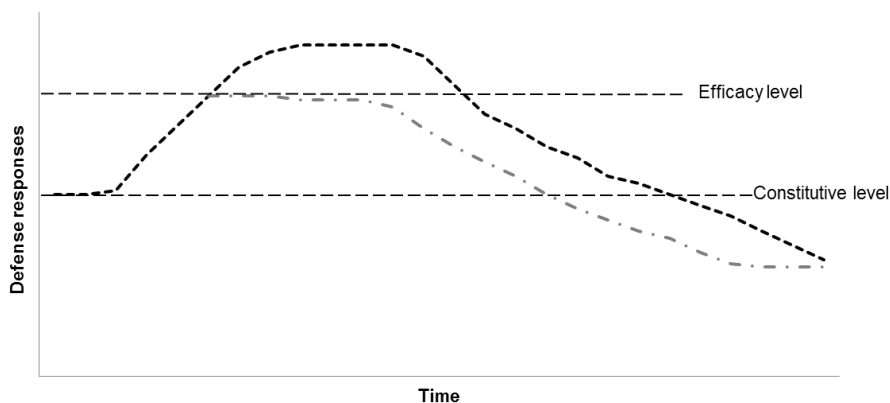

Figure 6.1 Hypothetical temporal change of induced defense after herbivory. Plants have a constitutive level of defense in the absence of herbivores. When plants are exposed to herbivory, induction of defense can lead to a higher level up to the level that deters or repels herbivore feeding (efficacy level, grey line). Depending on the amount of herbivore damage or herbivore species that causes the induction, a higher level of defense (than the efficacy level) may be reached (dark line) to cope with future herbivores. In both cases, plants usually maintain the defense at or above the efficacy level for a period and begin to decline over time until returning to the constitutive level or even a lower level (induced susceptibility).

Consequences of association with BG symbionts for dynamics of AG plant defenses

Mycorrhizal fungi can either directly induce plant defense (Gange and West 1994) or prime plants to show a quicker or stronger defense response against

subsequently-arriving herbivores (Pozo and Azcón-Aguilar 2007; Jung et al. 2012). A stronger defense can kill herbivores or retard their growth while an early-expressed defense can constrain herbivore growth and reproduction, leading to lower population growth in a longer run. Consequently, I first hypothesized that mycorrhization could result in higher mortality or lower growth rate of herbivores following previous induction. I observed lower growth rates of *S. exigua* on mycorrhizal plants (Figure 3.2) showing a higher defense level than non-mycorrhizal plants (reviewed by Koricheva et al. 2009).

As AMF can prime plants and provide plants with a quicker defense upon herbivory, I recorded the time course of induced defense in mycorrhizal and non-mycorrhizal plants. I hypothesized that induced defense can be expressed earlier after the onset of herbivory in mycorrhizal than in non-mycorrhizal plants. The results showed that, contrary to my hypothesis, the level of the plant defense compound catalpol linearly increased over time after induction but only in absence of AMF (Figure 3.3). These results suggest that mycorrhizal colonization can suppress further synthesis or accumulation of foliar defense compounds upon herbivory in *P. lanceolata* when these defense compounds are constitutively high in mycorrhizal plants (Figure 3.3). I noted that mycorrhization *per se* could systemically induce defenses in *P. lanceolata* foliage (Gange and West 1994) although my study also showed that a higher level of defense compounds may not necessarily result in a reduced performance of herbivores. For example, the higher level of constitutive catalpol in mycorrhizal plants did not correspond with a lower growth rate of herbivores (Figure 3.2 and 3.3). Instead, the decrease of food consumption by later herbivores was accompanied by an increase in catalpol over time but only in non-mycorrhizal plants (Figure 3.2, 3.3). Such findings suggest that AMF colonization may affect digestion of secondary defense compounds by insect herbivores and mitigate their negative effects on these herbivores in my research.

Usually AMF are considered to be plant-mutualists that can provide plants with nutrients and water, and that need carbon resources from the plant in return to support their own growth (Smith and Read 2010). My study and

several others shows that root colonization by AMF may also lead to neutral or negative influences on plants (Johnson et al. 1997; Klironomos 2003; Johnson et al. 2015) depending on both the abiotic (Johnson et al. 2015) and biotic (Pineda et al. 2013) conditions that the plant and AMF encounter. AMF colonization may not only alter plant defense against herbivores but also contribute to a plant's tolerance to herbivory though different mechanisms are involved in the two defense strategies (Tao et al. 2016). In this thesis I found that AMF greatly constrained plant growth and reduced plant biomass in the presence of secondary herbivores (chapter 3). Such results may demonstrate that AMF colonization may provide plants with a higher defense at the expense of plant growth that is otherwise favoring tolerance to herbivores.

Plant age matters in plant growth responses to AG and BG damages

The first two data chapters in this thesis focused on the effects of timing of herbivore attacks on plant defense against herbivores within the context of above-belowground interactions (chapters 2 and 3). They showed that AG and BG herbivore interactions via induced plant defenses have a temporal dimension and that other belowground organisms such as AMF may also participate in these interactions. Plants may experience variable levels of herbivory when they grow and their defense strategies, either tolerance or resistance, can vary during plant ontogeny (Root 1996). Plant age is frequently shown to affect a plant's adaptation to various environmental stresses (Boege and Marquis 2005) as it influences the plant's response in terms of induced defense (Quintero and Bowers 2011) and tolerance (Barton 2013) to stress. For example, defenses of young plants are often more easily inducible (Quintero and Bowers 2011) but young plants have lower tolerance (Elger et al. 2009). Relatively few studies, so far, have investigated both plant induced defense and tolerance responses in plants that vary in age. In chapter 4 I tested the hypothesis that young plants have higher levels of defense compounds but lower tolerance when exposed to AG or BG herbivory than old plants. I chose a grass species *Holcus lanatus*, which showed both defense and tolerance responses when exposed to herbivory. I used an approach comparing the defense and tolerance responses of *H. lanatus* following mechanical

defoliation and root herbivory by nematodes at various ontogenetic stages. The results showed that plants had lower levels of total phenolics and higher levels of %N in regrown foliage following defoliation, but that these responses were independent of plant age at defoliation. Such enhancement of foliar palatability induced by defoliation often facilitates contemporary herbivory and attracts more future herbivores. However, if these plants are damage-tolerant species such as grasses (Caldwell et al. 1981) they may eventually benefit from the enhanced plant quality by over-compensating defoliated tissues (McNaughton 1983; Paige and Whitham 1987; Agrawal 2000b). In chapter 4 I showed that the enhancement of foliar N by defoliation was only present in plants defoliated at intermediate and old age. It suggests that young plants may not be as capable as older plants in nutrient acquisition for tissue regrowth due to their smaller roots.

In addition to induced defense, I investigated plant relative growth responses to defoliation and root herbivory at variable plant ages to evaluate plant tolerances to these treatments. Given that plant tolerance usually increases with plant age as a plant's reserves increase over time (Boege et al. 2007) I hypothesized that plant growth rate was higher when plants had been exposed to defoliation or root herbivory at older ages. I measured plant growth rate as the biomass gain relative to the biomass present in a specific period after AG and BG herbivory. The results showed that plants tended to regrow removed shoot biomass only shortly after defoliation and that this level of regrowth decreased over time following defoliation. I attributed the results to a transient sink strength created by defoliation. Plant assimilates are preferably oriented towards carbon sink in shoots to establish plant photosynthesis as a response to defoliation (Briske and Richards 1995) until the sink decays as photosynthesis restored over time after defoliation. However, at the plant root level plants exposed to defoliation and root herbivory at older ages tended to invest less in root regrowth than at young and intermediate ages. It indicates that old plants may show a lower tolerance to defoliation and root herbivory. Such results contradict with other studies showing old plants are better in tolerance to defoliation. The inconsistences in plant growth to herbivory suggest that plants at different age differ in

allocation of assimilated resources to above- or belowground tissues for regrowth.

Plant age in relation to AG and BG herbivore populations

Defoliation can systemically induce defense compounds in roots and consequently constrain population growth of root herbivores (van Dam and Heil 2011). On the other hand, it can also initiate a reallocation of available resources to invest in root growth rather than in root defense (Wardle 2002) and this can facilitate root herbivores. The initiation of these two opposite processes are both dependent on plant age at defoliation (Quintero and Bowers 2011; Quintero et al. 2014). In my thesis I demonstrated that defoliation of *H. lanatus* did not alter measured aspects of plant root quality (concentration of total phenolics and N) at any plant age (chapter 4), and this suggest that plant defense compounds may not have played a major role in regulating populations of root herbivores. Instead, resource allocation to roots initiated by defoliation may have influenced root herbivore responses. In chapter 5 I hypothesized that defoliation could increase root-feeding nematode populations due to its positive effect on root biomass. In accordance with this hypothesis, I showed that defoliated plants sustained higher abundances of nematodes.

Since other work has shown that young plants tend to reduce the allocation of biomass to roots following defoliation (Hanley and Fegan 2007) I hypothesized that the positive effect of defoliation on root-feeding nematodes would be strongest in old plants. In contrast, I found that the positive effect of defoliation on nematodes was independent of plant age at defoliation (Figure 5.3). It suggests that the abundance of root-feeding nematodes in response to defoliation may not depend on root size but on other plant attributes initiated by defoliation. For example, root-feeding nematodes can be facilitated by an enhanced sink strength in roots due to defoliation (Kaplan et al. 2008b) because defoliation can redirect the flow of assimilates within the plant (Denno and Kaplan 2007), e.g. from shoots to roots in this case. On the other hand, the population dynamics of nematodes following defoliation was

independent of plant age at defoliation, which may suggest that the allocation of assimilates to roots following defoliation in *H. lanatus* plants is not plant-age specific either.

To quantitatively record plant defense a measure of herbivore performance can be more appropriate than measurements of nutrient value and concentration of defense compounds (Karban and Baldwin 1997). In my previous study I measured the concentration of total phenolics and carbon and nitrogen in *H. lanatus* roots that may provide an indication of the food quality experienced by the herbivores. However, neither of the chemicals in plant roots was altered by defoliation or changed with plant age, suggesting that food quality may not have been changed by defoliation. In contrast, the percentage of *Pratylenchus penetrans* remaining in the roots compared to that present in the soil, which is an indication of favorable root conditions, decreased with plant age. This indirectly suggests that living conditions of root-feeding nematodes decreased with plant age. Other characteristics of the roots or other root chemicals that were not measured, such as changes in the composition of phenolics (Erb et al. 2015) and primary compounds (amino acids, carbohydrates, etc.) or silica content (McNaughton et al.1985), may change with plant age. Whether these compounds can explain the changes in the herbivore populations is awaiting further test.

Conclusions and future studies

Conclusions

Induced plant defense and tolerance by herbivory are both widely acknowledged as major effective resistance strategies (Agrawal 1998; Strauss and Agrawal 1999). Their effectiveness depends on external factors such as the feeding location of the herbivores, the amount of damage inflicted by the herbivore, and herbivore diet specialization. Intrinsic factors including plant growth rate and resource allocation patterns can also influence a plant's resistance against herbivores. These factors interact to shape specific plant-herbivore relationships that usually vary in space and time. A variety of

studies have investigated spatially-separated herbivore interactions mediated by plant responses to these herbivores, for example AG-BG herbivore interactions. However, these studies often record the temporal dynamics of induced resistance following one time point at damage (e.g. van Dam and Raaijmakers 2006). Otherwise, they only explored these dynamics locally, for example in the AG compartment (Mathur et al. 2011). These studies ignore the fact that plants interact with multiple temporally- and spatially-separated herbivores. Consequently, the attempts of interpreting and predicting plant-herbivore interactions may be biased. Without recording the dynamics of these interactions the attempts are only snapshots and lack ecological realism. The current thesis examined the role of plant induced defense and tolerance in above-belowground herbivore interactions using a temporal approach. In the light of key findings in this thesis I conclude that:

1. The timing of when herbivory occurs at variable temporal scales can influence interactions among AG, BG herbivores and root symbionts. The direction and magnitude may vary with plant species, herbivore feeding modes and location of damages. Some findings contradict earlier studies that were conducted using similar approaches. For example, in my work prior AG herbivory tended to enhance BG herbivore performance whereas opposite results have been reported in other systems (Erb et al. 2011; Erb et al. 2015). A meta-analysis showed that BG herbivores facilitate AG herbivores only if they simultaneously colonize the plants (reviewed by Johnson et al. 2012) but in my work BG herbivores reduced food consumption of AG herbivore independent of whether they arrived before, at the same time or after the aboveground herbivore on the plant. Hence, my thesis highlights the importance of timing of herbivory for above-belowground interactions, but also acknowledges that these effects may be highly context dependent.

2. AMF colonization systemically increased foliar plant defense compounds but repressed its further increases upon AG herbivory. Such effects of AMF on plant defense may not necessarily benefit

plant fitness via e.g. diverting resources to plant constitutive defense, but via interfering with the further inducibility of these defenses (Karban and Baldwin 1997). In the presence of AMF, the performance of later arriving herbivores was reduced. These opposite roles of AMF in plant-herbivore relations observed in this thesis suggest that not only contemporary plant herbivore relations, but also future plant-herbivores regimes should be taken into account in understanding ecological functions such as plant defenses.

3. Combined AG and BG herbivory in *H. lanatus* enhanced aspects of foliar quality such as a decreased concentration of defense compounds and an increased concentration of N, potentially benefiting future herbivores. Such induced susceptibility was also observed when *P. lanceolata* was exposed to root feeding wireworms (chapter 2). Further, defoliation can shape root growth of which the direction and magnitude depend on the timing of defoliation. Plant roots tended to be less negatively affected by defoliation if defoliation occurred at older plant age. Since plants prioritize resources to defend the most valuable or most vulnerable organs ("optimal defense theory", Mckey 1974; 1979), defense or regrowth of root systems of perennial plants may be more important than to regrow the defoliated tissues when the plant is old.

4. Defoliation resulted in a new plant-herbivore interaction in relation to compensatory plant growth in *H. lanatus*. Since plants can better grow root systems following defoliation at old age they may thus sustain a higher abundance of root-feeding nematodes. Indeed defoliation overall enhanced the abundance of root-feeding nematode *P. penetrans*, but the enhancement was independent of plant age at defoliation. Thus, responses of root-feeding nematodes to defoliation may not result from an alteration of root quantity available to herbivores. The higher percentage of *P. penetrans* remaining in plant roots rather than in soil at younger plant age indirectly indicates that plant quality may be higher at this

developmental stage, which can affect the dynamics of nematode populations. Population densities of the competing species *Tylenchorhynchus dubius* was not altered by defoliation or plant age, suggesting a different response of this root herbivore species to altered plant traits.

Future studies

My thesis investigated aboveground-belowground herbivore interactions in relation to timing of herbivory at the individual and population level. Induced defense and tolerance were examined to interpret the patterns of interactions between organisms in AG and BG plant sections. The importance of timing in herbivory was highlighted in above-belowground herbivore interactions. However, the work presented here is limited to simplified interactions between single plant and herbivore species under controlled conditions. Investigation of consequences of AG-BG interactions for herbivore performance, population density and distribution needs further work at larger temporal scales so as to eventually contribute to insight in consequences of agricultural or ecological importance. To achieve this I propose several research directions that should be prioritized in future above-belowground herbivore interactions:

1. More plant and herbivore species can be included to assess the temporal pattern of each interaction within a more ecologically realistic AG-BG context. Higher complexity should be urged to model the relative roles of each player in a food web within a community.

2. AG and BG organisms that have other ecological niches, such as decomposers and predators, should also be included in AG-BG linkages. These organisms have profound impacts in influencing above-belowground herbivore interactions. For example, microbial communities can alter soil nutrient availability to plants and affect plant defense against herbivores. Predators or parasitoids can also highly control herbivore load on plants by e.g. cascading effects.

3. Timing of herbivory can shift plant-herbivore interactions both at the individual and the population level. The shift of these interactions can be exploited to control plant and herbivore species such as in invasive biology research.

4. Not only the concentration but also the composition of defense compounds affects the outcome of plant-herbivore interactions. Hence, the profile of defense compounds in plants should be measured to better predict the interactions among herbivores on that plant.

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Summary

Understanding of aboveground (AG) and belowground (BG) herbivore interactions has substantially advanced over the last decades. AG and BG herbivores are spatially separated, but can interact via their shared host plant. Foliar damage by AG herbivores can cause changes in root growth or root chemistry, which in turn can affect the performance of BG herbivores, and *vice versa*. The outcome of AG-BG herbivore interactions can be mediated by many factors, including the relative timing of herbivory and the interaction of plants with other non-herbivorous organisms, such as mycorrhizal fungi. The majority of previous studies has focused on these interactions at one moment in time, for example initiating defense using herbivory in one plant compartment and measuring the responses of herbivores in the other plant compartment at the same time. These studies ignore the fact that the interactions between AG and BG herbivores in reality can be dynamic in time.

Whether a herbivore will be affected by induced plant defense depends on the level of the defense at the time the herbivore feeds from the plant and the rate at which the induction changes over time. Via induced defense, a herbivore that feeds from a plant may influence its own food quality and hence its own performance, as well as that of other herbivores that arrive later. During ontogenetic development, plants can vary in their capacity of defense induction, so that the effects of herbivory on plant defense induction will depend on plant development stage. Plant defense *per se* is not constant over time and results in variation in plant defense induction by herbivory depending on development stage. Hence, studies on AG-BG herbivore interactions that do not take the timing of herbivory into account may provide an incomplete view of these interactions and this may hamper the prediction and interpretation of herbivore responses. In this thesis, I exposed plants to AG and BG herbivory at variable times and examined the responses of later arriving herbivores at both the individual and the population level. In addition, I also determined how the responses of AG herbivores can be mediated by the colonization of plant roots by symbiotic arbuscular mycorrhizal fungi (AMF).

In this thesis, *Plantago lanceolata* and *Holcus lanatus* were used as model plant species. *P. lanceolata* is a perennial forb that is widely spread across Europe and is frequently used in plant-insect interaction studies. This species produces several classes of secondary metabolites that can be induced by herbivores, of which iridoid glycosides (IGs) are one important class. The main compounds in the IG class are aucubin and catalpol that can be toxic and act as deterrents to various generalist herbivores, yet they can also be used as feeding or oviposition stimuli by specialists. *P. lanceolata* can be colonized by a variety of AMF species and is often employed to study plant-mycorrhizae interactions. As AMF can be strongly involved in the induction of plant defense, a three-way interaction among plant, insects and AMF has been investigated in this thesis. *Holcus lanatus* is a perennial and relatively fast-growing grass species that commonly occurs in temperate grasslands across Europe. It is frequently grazed by ungulates aboveground and hosts a number of root-feeding nematode species belowground. In the current thesis I used *P. lanceolata* to study its defense responses to timing of AG and BG herbivory, and investigate roles of AMF in mediating these responses. *H. lanatus* was used to detect the growth and defense responses to timing of aboveground foliage removal and I related this to the population dynamics of root-feeding nematodes.

The aim of this thesis was to analyze the importance of timing of herbivory in plant-herbivore interactions from an AG-BG perspective. Previous work suggests that AG and BG herbivores can greatly differ both in inducing defenses and in their responses to plant defense induction. Plant defense usually takes time to become effective and is not maintained continuously at a high level. Hence, the effectiveness and location of induced defense in a plant can change over time. Therefore, I tested the overall hypothesis that the direction and magnitude of impacts of plant defense induced by one herbivore on other herbivores not only depends on where the herbivore attacks the plants (AG or BG), but also on when the first herbivory event occurs relative to the second. I expected that the level of plant defense compounds in root and shoot tissues would differ when plants were exposed to various timing of AG and BG herbivory and that defense levels first increase and then decrease as time progresses after herbivory. Alternatively,

plants may not show an increase in the level of defense compounds after the first event of AG or BG herbivory but can be primed by these herbivores to express a more rapid or stronger defense when plants are exposed to herbivory at a later stage. Mycorrhizal fungi can also stimulate induction of plant defense and can prime plants so that plants have higher concentrations or a more rapid increase of defense compounds after herbivory.

I first investigated the effects of sequence of AG and BG herbivore arrival at plants on the level of induced plant defense and on the performance of these herbivores (chapter 2). *Spodoptera exigua* caterpillars and *Agriotes lineatus* wireworms were used as AG and BG herbivore, respectively. I exposed *P. lanceolata* plants to AG herbivory prior to, simultaneously with, or after the introduction of the BG herbivore, and *verse versa* and measured performance of the BG and AG herbivores. Opposite to most other studies, AG herbivory tended to facilitate the growth of the BG herbivores, but only when it preceded the arrival of the BG herbivore on plants. BG herbivory reduced food consumption by AG herbivores, but this effect was observed irrespective of the timing of arrival of the BG herbivore. AG and BG herbivory did not induce changes in IGs in plant roots and shoots respectively, so I attributed these effects to other unknown attributes that can be systemically induced by herbivory. An intriguing finding in this study was that AG herbivores when added alone reduced the food consumption and weight gain of their later arriving conspecifics, but the reduction disappeared if BG herbivores had been simultaneously introduced with the inducing AG herbivores. It suggests that the occurrence of BG herbivores may suppress the induction of defense by AG herbivory in plant foliage. The results of this study show that the sequence of herbivore arrival can determine the outcome of AG-BG interactions.

In chapter 3, I investigated how the AMF species *Funneliformis mosseae* influenced the induction of plant defense compounds by an AG herbivore at different time points following exposure to the herbivore. Plants often show a primed state with AMF colonization in roots and then express a quicker or stronger induced defense upon exposure to herbivory. I tested the hypothesis that mycorrhizal plants would be faster in their response to herbivory and

have higher levels of defense compounds following herbivory than non-mycorrhizal plants. In particular, I examined how the time of exposure of the plants to the AG herbivores influenced the rate and amount of defense induction in the plant as well as the performance of later arriving AG herbivores, and how this was modulated by AMF colonization. In line with my hypothesis, AG herbivores feeding on mycorrhizal plants overall had a lower growth rate than herbivores feeding on non-mycorrhizal plants. The time course of induced plant defense showed that growth rates of the later arriving herbivore decreased as time progressed between initial induction and the second herbivory event by the later herbivore (a period of 0 to 8 days in my study). The decrease of herbivore growth corresponded with a gradual increase of catalpol over this period, but this was only observed in non-mycorrhizal plants. Mycorrhizae may have suppressed further production of defense compounds in the plant because mycorrhizal plants had already higher levels of defenses prior to induction. Colonization of mycorrhizal fungi did not only enhance plant defense levels but also reduced plant biomass in this study. Hence the higher level of defense in mycorrhizal plants may be synthesized or accumulated at the expense of resources that otherwise can be used for other plant functions such as growth or storage.

In chapter 4, I examined the impacts of timing of removing shoots, as a proxy for aboveground herbivory, on plant growth and defense. Plant ontogeny can be an important determinant in responses of plant defense and tolerance to herbivory, because plants of various ages can differ in defense inducibility and growth rates. In this chapter, *H. lanatus* plants were subjected to aboveground clipping and belowground nematode exposure at variable ages and I recorded their chemistry and growth responses. I hypothesized that young plants have a higher level of defense, but a lower regrowth rate than older plants when exposed to AG clipping and/or BG herbivory. Plants regrew AG biomass removal by producing more shoots, but only shortly after defoliation, and had lower concentrations of total phenolics and higher concentrations of N in the regrown foliage. However, these plant responses did not depend on plant age at defoliation. However, I observed a belowground age-specific response in plant root growth after defoliation. Old defoliated plants did not grow roots after defoliation in comparison to plants

that were defoliated at younger age, indicating that old plants may be inferior to young and intermediate aged plants in root growth after defoliation. Plant biomass was not influenced by root herbivory by nematodes at any age. This study suggests that *H. lanatus* may show contrasting regrowth and defense responses depending on the timing of herbivory.

In chapter 5, I recorded population dynamics of root-feeding nematodes on *H. lanatus* plants that were defoliated at variable ages. Young plants were inoculated with a migratory endoparasitic nematode species *Pratylenchus penetrans* and an ectoparasitic species *Tylenchorhynchus dubius*, followed by an event of defoliation at young, intermediate and old plant age. I hypothesized that defoliation would benefit population growth of root-feeding nematodes and that the benefits would be strongest in old plants. The results were supporting the hypothesis in part by showing an overall positive effect of defoliation on *P. penetrans* populations. However, *T. dubius* populations were not affected, which is not in support of my hypothesis. My results suggest that population growth of root-feeding nematodes in response to plant defoliation may be species-specific. Interestingly, results from chapter 4 suggested that root quality did not change with plant age, but in chapter 5 the percentage of nematodes of the species *P. penetrans* remaining in roots rather than in soil was higher in young plants than in intermediate and old aged plants. It indirectly suggests that root quality available to nematodes other than concentration of N and total phenolics tended to decrease as plants aged. Combining all these results, this study suggests that plant-nematode interactions are influenced by aboveground events such as clipping. However, these interactions may not operate via induced changes in concentration of plant N and total phenolics that did not change with plant age or defoliation.

In my thesis I used a temporal approach to linking dynamics of AG-BG herbivore interactions with induced plant defense and tolerance. My thesis demonstrates that plant and herbivore performances can be altered by the timing of herbivory and this was particularly obvious for responses at the individual herbivore level. I conclude that the outcome of AG-BG herbivore interactions depends on the arrival time of the herbivores that modulate plant defense and tolerance, but also on the presence of other organisms such as

AMF that prime these plants. My thesis highlights the importance of timing of herbivory in assessing herbivore interactions and plant adaptations to these interactions in an AG-BG context. An understanding of AG-BG herbivore interactions from a temporal perspective can be crucial in predicting consequences such as plant damage or outbreaks of herbivores. Ultimately, my results may also contribute to developing novel strategies in the application of biocontrol of pests in agriculture.

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sacrificed a huge amount of time which you otherwise could have spent with your family. Our discussions on that experiment promote my thinking towards soil more and the knowledge out of these discussions definitely opened a window for my future direction of research. By the way, you really made great food, which convinces me European food can also be extremely good.

Regarding my journey in this lovely country, I have to mention Stijn, my best friend in the Netherlands. You really colored my life here and I cannot image how it would look like without your company. You are always ready to help me so that I even relied on you for almost everything. I like our talks a lot on the way to Orion for lunch because it can effectively relieve my regular daily stresses. We also had many dinners and drinks at which you always listened to me and gave me suggestions to get more joys from 'everyday life'. Stijn, you are really the most special and non-typical Dutch. You are kind of my 'yet another' supervisor that guided my life in Wageningen on the right way. As my best friend here, we did almost everything together, except travelling. I hope we can do this in China or somewhere one day to fulfill my appreciation on you. I am sure I will miss you a lot when I leave.

Sigrid, Rutger and Stefan, we were a team in *Dezier* (officially written as café 'De Zaaier') to talk, comment, make jokes etc., all these moments composed of my happiness after a day of work. So many funny things at your places, such as the vegetarian cake from a basement (Rutger), the robot and the lovely kid Mads (Stefan), the lost cat (Sigrid) etc. I cannot forget you for all you brought to me.

Haikun, you are a lovely girl that can obtain a lot of compliments from people. You look always happy, together with Stijn and Sigrid we had a good time in our office, also at your home with your mother talking about Chinese cultures and history. We shared a supervisor (you know who) for which we also had other topics to talk about. Every time I saw you, I felt much younger because of the love on life and passion to new challenges you showed.

Besides Haikun, we of course have a huge NIOO-Chinese community which also brought me so much fun. Sui, Jing/Steven, Jingying, Jinghua, Minghui, Kong, Yani, Cong, Peiyu, Chunxu, Libin, Wei, Baoyan, Yiying etc. all of you used your own way to shine my life. We had dinner together, organised activities together, watched movies together etc. and all these times are carved

in my memory. Sometimes I tell myself that NIOO is really an amazing place where all the best Chinese boys and girls are gathered. Among these friends, Sui needs to be highlighted because we really had a lot to share in the past years. Because of you, I started to play badminton which became my favorite sport now. We cooked together so many times, met so many Chinese visiting scholars, sang homemade KTV, and did countless other silly things. I never complimented you in words, but I have to say here that you are really talented in almost everything.

Taia, Olga, Moniek, and Martine, you are the first group of colleagues that I got to know when I arrived at this unfamiliar country. It is you who taught me how to get along with local people and how to deal with problems in European ways. I learned so much from you, starting from using forks and knives and cutting breads. You four told me the Dutch speed in biking. Really nice to remember the time spent with you. Also Roeland, you impressed me using your crazy ideas and talents in music. I do not forget our promises that we would travel in China one day. 'Fytotron team', Gregor, Roel, and Eke, I have to say you are so excellent in organising, managing and helping that without you my work in NIOO would have been much harder.

Of course, my other NIOO colleagues should also be listed here because you all together created such a comfortable working environment. Julie, Veronica, Ciska V, Ciska R, Thomas, Jasper, Sabrina, Marta, Feng, Kadri, Viola, Gerard, Elly, Gera, Jeff, Koen, Freddy, Tanja, Carla, Elly, Raquel, Kevin, Florentine, Rebecca, Paolo, Ruth, Sven, Mandy, Nico, Gerda, Pakize etc. Thank you all for giving me all kinds of knowledge and fun, providing me so many possibilities in my future research career.

I also give my thanks to some Chinese visiting scholars that I met either in NIOO or in University. Wei-bin, you helped me so much and you are really a nice friend to get along with. Together with Sui, we played badminton, cooked noodles, cycled, etc. Other scholars like Tieshan Xu, Lihong Gu, Yanling Hao, Sha Xue, Laiye Qu, Naili Zhang, Qi Li, Kai Cheng, Xingchun Tang, Jianming Bian, Qiufang Zhang etc. also contributed to my PhD in different ways. I am so glad that we all keep in touch and it is a fortune to know you all.

In the University, there is also a group of nice people who participated in my life in Wageningen. These friends include: Zhang Chen, Wu Jingbin, Xie Li,

Shi Wenbiao, He Jun, Stijn S, Siyu, Cheng Xu, Zeng Tian, Xiao Tingting, Defeng Shen, Qin Wei, Li Hucheng, Yang Xiaomei etc. I could not list all the names here but I do remember all the moments we shared.

Finally, I wanted to give all my thanks to my parents and Fengjiao. It is all your support which lead me to this PhD. Fengjiao, you are the biggest gift I had in Wageningen. Your presence shed lights on my everyday here which is a strong favor for my PhD and my future life. I am a lucky guy to meet you, to have your selfless support and to be companied whenever I suffered difficulties. We together spend two years in this small city and if possible I hope we could experience more hand in hand for future.

All my dear colleagues, friends, and beloves thank you so much and I begin to miss you all already.

Minggang Wang, in Wageningen

Curriculum vitae

Minggang Wang was born on the 20th of November 1984 in Xinyang, China. After high-school study at hometown, he studied agronomy at Henan University of Science and Technology where he obtained his bachelor degree in 2008. He then moved to Xi'an to continue his studies at Northwest A&F University and got a master degree on plant pathology at 2011. His master thesis, titled "Isolation of phyllosphere actinomycetes from cucumber and biocontrol on cucumber grey mold and target leaf spot" witnessed his strong interest on plant-organism interactions. To further explore this interest, he moved to the Netherlands funded by China Scholarship Council (CSC) to persue a doctor degree at Septermber in 2011 under supervisions by Prof. Wim H. van der Putten, Dr. T. Martijn Bezemer and Dr. Arjen Biere at Department of Terrestrial Ecology, Netherland Institute of Ecology. By collaboration with his colleagues, he conducted a project on timing of above-belowground interactions, which resulted in this thesis.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)
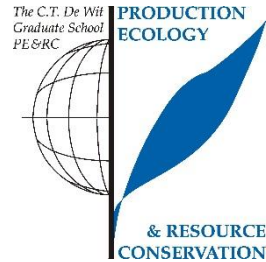
Review of literature (6 ECTS)
- Temporal aspect of above-belowground interactions

Writing of project proposal (4.5 ECTS)
- Temporal aspect of above-belowground interactions

Post-graduate courses (3 ECTS)
- Multivariate analysis; PE&RC (2012)
- Soil ecology; PE&RC (2012)

Laboratory training and working visits (4.5 ECTS)
- Identification of terrestrial and freshwater nematodes; Laboratory of Nematology, WUR (2012)
- International training course on identification on soil animals; Swedish Agricultural University (2014)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Insects: above-belowground interactions (2015)

Deficiency, refresh, brush-up courses (1.5 ECTS)
- Basic statistics; PE&RC (2012)

Competence strengthening / skills courses (2 ECTS)
- Stress identification & management; WGS (2014)
- Reviewing a scientific paper; WGS (2015)
- Scientific writing; WGS (2015)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC Day (2012)
- PE&RC Weekends (2012, 2014, 2015)
- Soil-vegetation interaction: from Rhizosphere to ecosystem (2014)

Discussion groups / local seminars / other scientific meetings (3.6 ECTS)
- Wageningen evolution & ecology seminar (2013, 2014)
- Centre for Soil Ecology (CSE) meetings (2014)
- Centre for Soil Ecology for annual meeting (2015)

International symposia, workshops and conferences (8.6 ECTS)
- Multitrophic interaction; Göttingen, Germany (2012)
- Netherlands Annual Ecology Meeting (NAEM); Lunteren, the Netherlands (2012, 2014)
- Netherlands Entomology Day; Ede, the Netherlands (2013, 2015)
- BES/SFE Annual meeting; Lille, France (2014)